Docket No. 44559

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: Jeffrey M. Isner EXAMINER: Low. C.

SERIAL NO. 08/318,045 GROUP: 1804

FILED: October 4, 1994

FOR: METHOD AND PRODUCTS FOR NUCLEIC ACID DELIVERY

THE HONORABLE COMMISSIONER
OF PATENTS AND TRADEMARKS
WASHINGTON, DC 20231

Sir:

DECLARATION OF DR. JEFFREY M. ISNER
UNDER 37 C.F.R. §132

I, Jeffrey M. Isner, hereby declare:

1. I am a citizen of the United States of America residing at 34 Brenton Road, Weston, Massachusetts 02193.

2. I graduated from University of Maryland in 1969 with a Bachelor of Science degree. In 1973 I received an MD degree from Tufts University School of Medicine. A copy of my curriculum vitae is attached hereto as Attachment A.

3. I am currently a professor of Medicine and Cardiology at Tufts University School of Medicine, Boston, Massachusetts, as well as Chief of Cardiovascular Research at St. Elizabeth's Medical Center, Boston, Massachusetts. I have held these positions since 1988.

4. I am also the Director of the Human Gene Therapy Laboratory at St. Elizabeth's Medical Center.

5. Attached to this Declaration as Attachment B is a manuscript recently submitted to the New England Journal of Medicine setting forth human clinical results seen with the present invention. This document accurately reflects work done under my supervision and control.

6. Briefly, in the study a hydrogel-coated balloon angioplasty catheter was used to perform percutaneous arterial gene transfer of phVEGF$_{165}$, a eukaryotic expression plasmid encoding the 165-amino acid isoform of vascular endothelial growth factor (VEGF), to a lower extremity arterial site. Patients received 100 μg (n = 1), 500 μg (n = 1), or 1000 μg (n = 5) of naked plasmid DNA without adjunctive vectors.

Of the five patients receiving 1000 μg of plasmid DNA, the following results were observed:

In one patient, (patient 4), gene transfer was performed immediately upstream from the site of a total occlusion. This was the only case in this study in which gene transfer was performed in an occluded vessel, and was the only patient in whom follow-up angiography disclosed compromised patency at the transfected site.

In three of the four other patients treated with 1000 μg of plasmid DNA in whom the gene transfer site remained unobstructed, evidence of augmented flow to the distal portion of the ischemic limb was documented by three independent tests.

1. Intravascular Doppler disclosed an increase in resting blood flow (132.3 to 188.5% of baseline), and maximum flow (120.0 to 158.6%), provoked by intra-arterial nitroglycerin.

2. Contrast-negative magnetic resonance angiography (MRA) graphically confirmed the increased blood flow in these three patients.

3. Contrast angiography documented accelerated delivery of contrast media from the common femoral artery to the pedal arch vessels in these three patients.

Moreover, these three patients, each of whom presented with several (6 to 17) months of ischemic rest pain, remain free of rest pain at greater than 3-months follow-up. In the fourth patient, in whom rest pain was associated with aggressive growth and the size of the ischemic ulcer during the three months prior to gene therapy, further extension of the ulcer was blunted for two months post-gene therapy. To my knowledge, this is the first therapy to relieve ischemic rest pain.

While these results are currently anectdotal in nature, they indicate that my method of nucleic acid delivery can and does work in providing therapeutic angiogenesis.

7. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 12/7/95

Jeffrey M. Isner

wp#40395


11A

Revised 11/1/95

CURRICULUM VITAE

JEFFREY MICHAEL ISNER, M.D.

Present Address:	34 Brenton Road, Weston, Massachusetts 02193
Telephone:	(H) 617-431-8460 (W) 617-789-2392 Fax 617-789-5029
Date of Birth:	December 11, 1947
Citizenship:	U.S.A.
Wife:	Linda Hajjar Isner
Children:	Joshua Michael, Jessica Marie, and Matthew Hajjar
Father:	Justin Isner, born Burghaslach, Germany, 11/14/10; died Canton, Ohio 3/3/75
Mother:	Lilli Isner, born Bamberg, Germany, 6/13/17

PRESENT POSITION

Professor of Medicine and Pathology, Tufts University School of Medicine, Boston, Massachusetts, 1988 to present

Chief, Cardiovascular Research, St. Elizabeth's Medical Center, Boston, Massachusetts, 1988 to present

Director, Human Gene Therapy Laboratory, St. Elizabeth's Medical Center, Boston, Massachusetts
- P.I. Arterial Gene Transfer for Therapeutic Angiogenesis; approved by RAC #9409-088FDA #5777 activated 12/7/94
- P.I. Arterial Gene Therapy for Restenosis (Currently pending final FDA approval)

Director, Core Pathology Laboratory for Framingham Heart Study, 1979 to present

Director, Core Pathology Laboratory for Study of Sudden Cardiac Death, in association with Medical Examiner, Dr. George Katsas, 1979 to 1989

Consultant, Department of Pathology, New England Medical Center, Boston, Massachusetts, 1988 to present

Research Affiliate, George R. Harrison Spectroscopy Laboratory, Massachusetts Institute of Technology, 1986 to present

Director,Tufts University School of Medicine 4th Year Medical Student Clerkship: Vascular Medicine

ADVISORY GROUPS

American College of Cardiology

Medical Devices Committee, American College of Cardiology (1986 - 1990)
Peripheral Vascular Disease Committee, American College of Cardiology (1991-1996), Chairman (1994-1997)
Abstract Reviewer, American College of Cardiology Annual Scientific Sessions (1988 - present)
Director, Heart House Post-Graduate Course, "Peripheral Vascular Disease: Current Strategies for Diagnosis and Therapy" (1992-present)
Government Relations Committee, Key Contacts 1993-present
Annual Scientific Session Program Committee, American College of Cardiology (1994-1996)

American Heart Association

Board of Directors, American Heart Association, Massachusetts Affiliate (1985 - 1986; 1988 - 1989; 1992-1993)
Research Peer Review Committee of the American Heart Association, Massachusetts Affiliate (1985 - 1991)
Research Policy Committee, American Heart Association, Massachusetts Affiliate (1991-1994).
Council on Clinical Cardiology Program Committee (1991-present; Vice Chairman, 1994-1995)
Abstract Reviewer, Angioplasty Section, American Heart Association Annual Scientific Sessions (1984-1989; 1991-present)

American Society for Lasers in Medicine and Surgery

Instrumentation Committee, American Society for Lasers in Medicine and Surgery (1987-1990)
Standards of Training and Development Committee, American Society for Lasers in Medicine and Surgery (1988-1991)
Chairman, Cardiovascular Subsection, Annual Scientific Sessions 1987, 1991
Advisory Panel to the Director of Continuing Medical Education, American Society for Laser Medicine and Surgery (1990)
Nominating Committee, American Society for Laser Medicine and Surgery (1992)

National Institutes of Health

Surgery and Bioengineering Study Section (NHLBI) (October, 1984)
SBIR Study Section (NIH) (March, 1985)
SBIR Study Section (NIH) (July, 1985)
SBIR Study Section (NIH) (October, 1987)
SBIR Study Section (NIH) (August, 1988)
Cardiovascular A Study Section (NHLBI) (March, 1990)
Cardiovascular A Study Section (NHLBI) (July 1, 1991-June 30, 1995)

Other

Referee, U.S. - Israel Binational Science Foundation
Referee, Medical Research Council of Canada
Referee, V.A. Merit Grants Program
Review Panel of MIT Laser Biomedical Research Center (1986 - 1988)
Thesis Reader, Harvard University-MIT Division of Health Sciences and Technology
External Advisory Committee, Atherosclerosis and Vascular Biology Training Center, Baylor College of Medicine, Houston, TX
Section Editor, "Vascular Biology and Medicine," Textbook of Cardiovascular Medicine, E. Topol, Ed., Lippincott, In Preparation

EDITORIAL BOARD

American Heart Journal
American Journal of Cardiac Imaging
American Journal of Cardiology
Cardiovascular Pathology
Catheterization and Cardiovascular Diagnosis
Circulation
Clinical Cardiology
Journal of the American College of Cardiology (1987-1990; 1993-present)
Journal of Invasive Cardiology
Journal of Vascular Medicine and Biology
Laser Medicine and Surgery News
Lasers in Surgery and Medicine
Trends in Cardiovascular Medicine (Executive Editor)

EDITORIAL CONSULTANT

American Journal of Pathology
American Journal of Physiology
Annals of Internal Medicine
Arteriosclerosis, Thrombosis, and Vascular Biology
Atherosclerosis
Cardiovascular Research
Catheterization and Cardiovascular Diagnosis
Circulation Research
Endothelium
European Heart Journal
Human Gene Therapy
Human Pathology
IEEE Journal of Quantum Electronics
Journal of the American Medical Association
Journal of Biological Chemistry
Journal of Cardiovascular Pathology
Journal of Cellular Physiology
Journal of Clinical Investigation
Journal of Vascular and Interventional Radiology
Mayo Clinic Proceedings
New England Journal of Medicine
Pediatrics
Proceedings of the National Academy of Sciences U.S.A.
Radiology
Science

HONORARY SOCIETIES

Phi Beta Kappa
American Society for Clinical Investigation

PROFESSIONAL SOCIETIES

American College of Cardiology (Fellow)
American Federation for Clinical Research
American Heart Association, Council on Clinical Cardiology (Fellow) and
Council on Atherosclerosis (Fellow)
American College of Physicians (Fellow)
American Society of Cardiovascular Interventionists
American Society for Laser Medicine and Surgery (Fellow)
New England Society of Pathologists
Society for Cardiac Angiography and Interventions (Fellow)
Society for Cardiovascular Pathology
Society for Vascular Medicine and Biology (Fellow)

GRANT SUPPORT AS PRINCIPAL INVESTIGATOR

National Institutes of Health
HL32747
Mechanisms of Cardiovascular Laser Phototherapy
12/1/85 - 11/30/88
Total direct costs for period of award: $436,305

National Institutes of Health
HL40518
Laser Irradiation and Vascular Reactivity
4/1/88 - 3/30/91
Total direct costs for period of award: $373,267

National Institutes of Health
HL40518
Laser Irradiation and Vascular Reactivity II
12/1/91-11/30/94
Total direct costs for period of award: 366,397

National Institutes of Health
HL 02824
Vascular Disease Academic Award
7/1/92-6/30/97
Total direct costs for period of award: $762,500

National Institutes of Health
HL53354
Therapeutic Angiogenesis in Vascular Medicine
12/1/94-11/30/99
Total direct costs for period of award: $555,612

Whitaker Foundation
Pulsed Laser Energy for Cardiovascular Therapy
2/1/86 - 1/31/89
Total costs for period of award: $150,000

Eleanor Naylor Dana Charitable Trust
Cardiovascular Applications of Laser Irradiation
4/87 - 3/90
Total costs for period of award: $300,000

DVI-Simpson Atherectomy Research Foundation
Use of Quantitative PCR to Study Nonmuscle Myosin Gene Expression in Human Atherosclerotic Lesions
7/25/92-7/24/93
Total costs for period of award: $25,000

POST-DOCTORAL FELLOWSHIP SUPPORT:

Lawrence J. Deckelbaum, M.D. u/d J. Isner
Mechanisms of Coronary Arterial Laser Phototherapy
American Heart Association, Massachusetts Affiliate
7/1/84 - 6/30/85

Constance D. Fields, M.D. u/d J. Isner
Mechanisms of Pulsed Laser Ablation in a Blood Field
American Heart Association, Massachusetts Affiliate
7/1/87 - 6/30/88

Saurabh Chokshi, M.D. u/d J. Isner
Effects of Laser Radiation and Ultrasound Energy on Vascular Reactivity
American Heart Association, Massachusetts Affiliate
7/1/88 - 6/30/90

Alexandra R. Lucas, M.D. u/d J. Isner
Pulsed Laser Energy Profile for Irradiation of Coronary Plaque
Alberta Heritage Foundation for Medical Research
9/1/87 - 9/1/89

P. Gabriel Steg, M.D. u/d J. Isner
Laser-Induced Changes in Vasomotion
French Government Trainee
12/86 - 4/87

Guy Leclerc, M.D. u/d J. Isner
Percutaneous Gene Therapy
Heart and Stroke Foundation of Canada
Recipient: First place, Young Investigator's Award, Society of Cardiovascular Pathology, 1990.
7/89-6/91

Morris Mosseri, M.D. u/d J. Isner
American Physician's Fellowship
8/89-8/91

J. Geoffrey Pickering, M.D., Ph.D. u/d J. Isner

Nonmuscle Myosin and Vascular Smooth Muscle Cell Proliferation
Medical Research Council of Canada
Recipient: First place, Young Investigator's Award, Canadian Cardiovascular Society, 1992.
7/1/91-6/30/93

Sigrid Nikol, M.D. u/d J. Isner
Analysis of Human Atheromata Obtained by Directional Atherectomy Using In Situ Hybridization and Immunohistochemistry
Deutsche Forschungsgemeinschaft (DFG)
7/91-6/92

Reimer Riessen, M.D. u/d J. Isner
Strategies for Percutaneous Arterial Gene Transfer
Deutsche Forschungsgemeinschaft (DFG)
7/92-6/93

Satoshi Takeshita, M.D. u/d J. Isner
Arterial Gene Transfer: Effect of Cell Proliferation on Transfection Efficiency
Recipient: Young Investigator's Award, AHA Biology of the Vascular Wall (Boston, 1993).
Recipient: Raymond Kalil Cardiovascular Research Award (1994).
10/91-9/94

Laurent Feldman, M.D.
Adenoviral Gene Transfer
Fulbright Scholar
10/93-10/94

Kathleen Hogan, M.D.
Evaluation of Therapeutic Angiogenesis with Vascular Endothelial Growth Factor (VEGF) in a Swine Model of Coronary Artery Occlusion
Recipient: Hewlett-Packard Medical Fellowship Award
7/95-6/96

BOARD CERTIFICATION

National Board of Medical Examiners
Diplomate, American Board of Internal Medicine - #55420 - 6/16/76
Diplomate, Subspecialty of Cardiovascular Disease - #55420 - 10/19/77

LICENSURE

California	#G40480
District of Columbia	#8695
Maryland	#D22287
Massachusetts	#45019

EDUCATION

B.S.	University of Maryland, College Park, Maryland, 1969, Magna Cum Laude
M.D.	Tufts University School of Medicine, Boston, Massachusetts, 1973.

POST-GRADUATE TRAINING

Internship	St. Elizabeth's Hospital, Boston, Massachusetts July, 1973 - June 1974 Fredrick Stohlman, M.D., Program Director
Residency	Georgetown University Hospital, Washington, D.C. July, 1974 - June, 1975 Dudley Jackson, M.D., Program Director
Fellowship in Cardiology	Georgetown University Hospital, Washington, D.C. Cardiology July, 1975 - June, 1977 W. Proctor Harvey, M.D., Program Director
Staff Associate	Pathology Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland (William C. Roberts, M.D., Branch Chief) July, 1977 - September, 1979.

OTHER PROFESSIONAL EXPERIENCE

Instructor, Department of Medicine, Georgetown University School of Medicine Washington, D.C., 1977 - 1979.

Assistant Professor of Medicine and Pathology, Tufts-New England Medical Center, Boston, Massachusetts, Oct., 1979 - March, 1983.

Associate Professor of Medicine and Pathology, Tufts University School of Medicine, Boston, Massachusetts, April 1983 - September, 1988.

Director, Core Pathology Laboratory for Nifedipine Acute Myocardial Infarction Study. (Drs. James E. Muller and Eugene Braunwald, Project Directors), 1979-1983.

Director, Core Pathology Laboratory, CAVEAT (Coronary Angioplasty versus Excisional Atherectomy Trial) 1991-1993

Consultant - Boston Veteran's Administration Hospital, 1979-1988.

Cardiologist, New England Lupus Center, 1981-1988.

Associate Director, Cardiac Catheterization and Permanent Pacemaker Laboratory, New England Medical Center, 1979-1988.

Director, Cardiac Pathology Laboratory, New England Medical Center, 1979-1988.

Medical School Committee for Evaluation of 3rd and 4th Year Medical Student Clerkships (1981-1983).

Publications, cont'd

41. Isner, J.M., Fortin, R.V.: Frequency in nonangioplasty patients of morphologic findings reported in coronary arteries treated with transluminal angioplasty. American Journal of Cardiology 51:689-693, 1983.

42. Roberts, W.C., Isner, J.M., Virmani, R.: Left ventricular incision midway between the mitral anulus and the stumps of the papillary muscles during mitral valve excision with or without rupture or aneurysmal formation: Analysis of 10 necropsy patients. American Heart Journal 104:1278-1287, 1982.

43. Salem, D.N., Isner, J.M., Hopkins, P., Konstam, M.A.: Ergonovine provocation in post-partum myocardial infarction. Angiology 35:110-114, 1984.

44. Isner, J.M., Konstam, M.A., Fortin, R.V., Salem, D.N.: Delayed thrombolysis of streptokinase-"resistant" occlusive thrombus: Documentation by pre- and post-mortem coronary angiography. American Journal of Cardiology 52:210-211, 1983.

45. Isner, J.M., Shen, E.M., Martin, E.T., Fortin, R.V.: Sudden unexpected death due to anomalous origin of the right coronary artery from the left sinus of Valsalva. American Journal of Medicine 76:155-158, 1984.

46. Isner, J.M., Harten, J.: Factitious lowering of serum potassium following cardiopulmonary resuscitation. Implications for evaluating arrhythmogenicity of hypokalemia. Archives of Internal Medicine 145:161-162, 1985.

47. Isner, J.M., Lee, S.S.: Clinicopathological Conference. New England Journal of Medicine 309:1233-1242, 1983.

48. Schmierer, J.A., Isner, J.M., Konstam, M.A., Salem, D.N.: Catheter fragmentation: a potential source of catheter embolus. Catheterization and Cardiovascular Diagnosis 9:595-599, 1983.

49. Isner, J.M., Salem, D.N.: The persistent enigma of percutaneous angioplasty. International Journal of Cardiology 6:391-400, 1984.

50. Isner, J.M.: The pathology of pacemakers. Intelligence Reports in Cardiac Pacing and Electrophysiology 2:2-6, 1983.

51. Donaldson, R.F., Isner, J.M.: Intercoronary continuity: an anatomic basis for bidirectional flow distinct from coronary collaterals. American Journal of Cardiology 53:351-352, 1984.

52. Salem, D.N., Konstam, M.A., Isner, J.M., Bonin, J.D.: Comparison of effects on left ventricular ejection fraction of iopamidol and renografin during left ventriculography and coronary arteriography. Investigative Radiology 19:S203-S205, 1984.

53. Konstam, M.A., Salem, D.N., Isner, J.M., Zile, M.R., Kahn, P.C., Bonin, J.D., Levine, H.J.: Vasodilator effect on right ventricular function in congestive heart failure and pulmonary hypertension: end-systolic pressure-volume relationship. American Journal of Cardiology 54:132-136, 1984.

54. Rifkin, R.D., Isner, J.M., Carter, B.L., Bankoff, M.S.: Combined postero-anterior subepicardial fat simulating the echo-cardiographic diagnosis of pericardial effusion. Journal of American College of Cardiology 3:1333-1339, 1984.

Publications, cont'd

55. Salem, D.N., Findlay S.R., Isner, J.M., Konstam, M.A., Cohen, P.: Comparison of histamine release effects of ionic and non-ionic radiographic contrast media. American Journal of Medicine 80:382-384, 1986.

56. Isner, J.M., Clarke, R.H., Pandian, N.G., Donaldson, R.F., Salem, D.N., Konstam, M.A., Payne, D.D., Cleveland, R.J.: Laser myoplasty for hypertrophic cardiomyopathy. Initial in-vitro experience in human postmortem hearts and in-vivo experience in a canine model (transarterial) and human patient (intra-operative). American Journal of Cardiology 53:1620-1626, 1984.

57. Isner, J.M., Michlewitz, H., Donaldson, R.F., Konstam, M.A., Salem, D.N.: Laser-assisted debridement of aortic valve calcium. American Heart Journal 109:448-452, 1985.

58. Isner, J.M., Clarke, R.H., Donaldson, R.F., Aharon, A.S.: Identification of photoproducts liberated by in vitro laser argon irradiation of atherosclerotic plaque, calcified cardiac valves, and myocardium. American Journal of Cardiology 55:1192-1196, 1985.

59. Sarno, R.C., Carter, B.L., Isner, J.M.: Computed tomographic demonstration of metastatic disease to the heart. Noninvasive Medical Imaging. 1:265-268, 1984.

60. Isner, J.M., Clarke, R.H.: Lasers: their potential in cardiovascular medicine. Cardiovascular Medicine 23-27, 1985.

61. Isner, J.M., Roberts, W.C., Heymsfield, S., Yager, J.: Anorexia nervosa and sudden death. Annals of Internal Medicine 102:49-52, 1985.

62. Geller, M.J., Isner, J.M., Payne, D.D., Salem, D.N.: Limb loss due to transvenous endocardial pacemaker therapy. American Journal of Medicine 78:351-354, 1985.

63. Konstam, M.A., Cohen, S.R., Salem, D.N., Isner, J.M., Das, D., Zile, M.R., Levine, H.J., Kahn, P.C.: Comparison of left and right ventricular end-systolic pressure-volume relations in congestive heart failure. Journal of the American College of Cardiology 5:1326-1334, 1985.

64. McCullough, K.I., Isner, J.M., Pandian, N.G., Bankoff, M.S.: Circulatory shock due to ascites and responsive to paracentesis. American Journal of Cardiology 56:500-501, 1985.

65. Salem, D.N., Hymanson, A., Isner, J.M., Bankoff, M.S., Konstam, M.A.: Congenital absence of the left pericardium: diagnosis by computed tomography. Catheterization and Cardiovascular Diagnosis 11:75-79, 1985.

66. Isner, J.M., Michlewitz, H., Clarke, R.H., Estes, N.A.M.E., Donaldson, R.F., Salem, D.N., Bahn, I.: Laser photoablation of pathologic endocardium. In vitro findings suggesting a new approach to the surgical treatment of refractory arrhythmias and restrictive cardiomyopathy. Annals of Thoracic Surgery. 39:201-206, 1985.

67. Isner, J.M., Clarke, R.H.: The current status of lasers in the treatment of cardiovascular disease. IEEE Journal of Quantum Electronics QE-20:1406-1420, 1984.

68. Deckelbaum, L.I., Isner, J.M., Konstam, M.A., Salem D.N.: Catheter-induced versus spontaneous spasm: do these coronary bedfellows deserve to be estranged? American Journal of Medicine 79:1-4, 1985.

Publications, cont'd

69. Pandian, N.G., Isner, J.M., McInerney, K.P., Caldeira, M.E., Donaldson, R.F.: Left atrial myxoma - implications of site size, mobility, and tissue structure. Echocardiography 2:113-118, 1985.

70. Cameron, J., Isner, J.M., Salem, D.N., Estes, N.A.M. III: Cardiac electrophysiologic testing: its role in the selection of antiarrhythmic drug regimens for supraventricular and ventricular arrhythmias. Pharmacotherapy 5:95-108, 1985.

71. Isner, J.M., Donaldson, R.F., Funai, J.T., Deckelbaum, L.I., Pandian, N.G., Clarke, R.H., Bernstein, J.S.: Factors contributing to perforations resulting from laser coronary angioplasty. Observations in an intact human post-mortem model of intra-operative laser coronary angioplasty. Circulation 72:II-191-199, 1985.

72. O'Donnell, T.F., Erdoes, L., Mackey, W.C., McCullough, J., Shepard, A., Heggerick, P., Isner, J.M., Callow, A.D.: Correlation of B-mode ultrasound imaging and arteriography with pathologic findings at carotid endarterectomy. Archives of Surgery 120:443-449, 1985.

73. Isner, J.M., Pandian, N.G., McInerny, K.P., Caldeira, M.E., Funai, J.T., Bojar, R.: The pericardial tourniquet: evaluation of the anatomic and physiologic features of constrictive pericarditis by combined use of computed tomography and cardiac ultrasound. Echocardiography 2:197-205, 1985.

74. Deckelbaum, L.I., Isner, J.M., Donaldson, R.F., Clarke, R.H., Laliberte, S., Aharon, A.S., Bernstein, J.S.: Reduction of pathologic tissue injury using pulsed energy delivery. American Journal of Cardiology 56:662-667, 1985.

75. Pandian, N.G., Isner, J.M., McInerney, K.P., Caldeira, M.E., Funai, J.T., Wang, S.S., Ramirez, A.: Non-invasive assessment of the complicated myocardial infarction: use of Doppler and two-dimensional echocardiography to differentiate ventricular septal rupture from rupture of mitral apparatus. Echocardiography 2:329-336, 1985.

76. Konstam, M.A., Hill, N.S., Bonin, J.D., Isner, J.M.: Prostaglandin mediation of hemodynamic responses to pulmonary microembolism in rabbits: effects of ibuprofen and meclofenamate. Experimental Lung Research 12:331-345, 1987.

77. Isner, J.M., Donaldson, R.F., Deckelbaum, L.I., Clarke, R.H., Laliberte, S.M., Ucci, A.A., Salem, D.N., Konstam, M.A.: The excimer laser: gross, light microscopic, and ultrastructural analysis of potential advantages for use in laser therapy of cardiovascular disease. Journal of the American College of Cardiology 6:1102-1109, 1985.

78. Simons, M., Isner, J.M., Pandian, N.G., Konstam, M.A.: Non-invasive diagnosis of cardiac amyloidsis. Echocardiography 2:401-408, 1985.

79. Deckelbaum, L.I., Isner, J.M., Donaldson, R.F., Laliberte, S.M., Clarke, R.H., Salem, D.N.: Use of pulsed energy delivery to minimize tissue injury resulting from carbon dioxide laser irradiation of cardiovascular tissues. Journal of the American College of Cardiology 7:898-908, 1986.

80. Isner, J.M., Clarke, R.H.: Laser angioplasty: unraveling the Gordian knot. Journal of the American College of Cardiology 7:705-708, 1986.

81. Keyes, T., Clarke, R.H., Isner, J.M.: Theory of photoablation and its implication for laser phototherapy. Journal of Physical Chemistry 89:4194-4196, 1985.

Publications, cont'd

82. Simons, M., Pandian, N.G., Isner, J.M., McInerney, K., Caldeira, M.: Calcific aortic stenosis. Echocardiography 2:481-490, 1985.

83. Isner, J.M.: Light in the heart. Tufts Medical Alumni Bulletin 46:12-13, 1985.

84. Salem, D.N., Konstam, M.A., Isner, J.M., Bonin, J.D.: Comparison of the electrocardiographic and hemodynamic responses to ionic and non-ionic radiocontrast media during left ventriculography: a randomized double-blind study. American Heart Journal 111:533-536, 1986.

85. Jansyn, E.M., Pandian N.G., Isner, J.M., McInerny, K.P., Caldeira, M.E.: Free-floating left atrial thrombus producing intermittent exacerbation of mitral valular stenosis. Echocardiography 3:47-53, 1986.

86. Isner, J.M., Clarke, R.H., Donaldson, R.F., Salem, D.N., Konstam, M.A., Payne, D.D., Levine, H.J., Cleveland, R.J.: Application of laser irradiation to coronary arterial recanalization. Il Nuovo Giornale Itliano Di Medicina 1:119-126, 1986.

87. Roberts, D.A., Isner, J.M., Deckelbaum, L.I., Konstam, M.A., Salem, D.N.: Acute myocardial infarction, normal coronary arteries, and catheter-induced spasm. American Journal of Cardiology 57:360-362, 1986.

88. Isner, J.M., Estes, N.A.M., Payne, M.D., Rastegar, H., Clarke, R.H., Cleveland, R.J.: Laser-assisted endocardiectomy for refractory ventricular tachyarrhythmias: preliminary intra-operative experience. Clinical Cardiology 10:201-204, 1987.

89. Isner, J.M., Donaldson, R.F., Fortin, A.H., Tischler, A., Clarke, R.H.: Attenuation of the media of coronary arteries in advanced atherosclerosis. American Journal of Cardiology 58:937-939, 1986.

90. Kurkjian, K., Naber, S., McInerney, K.P., Caldeira, M., Pandian, N.G., Isner, J.M.: Echocardiographic evaluation of metastatic pericardial heart disease. Echocardiography 3:273-280, 1986.

91. Isner, J.M.: Laser-assisted ventricular endocardiectomy. Clinical Laser Monthly 4:1S-3S, 1986.

92. Isner, J.M., Donaldson R.F., Fulton, D., Bahn, I., Payne D.D., Cleveland R.J.: Cystic medial necrosis in coarctation of the aorta: a potential factor contributing to adverse consequences observed following percutaneous balloon angioplasty of coarctation sites. Circulation 75:689-695, 1987.

93. Isner, J.M.: The cardiologist and the pathologist: the interactions of both and the limitations of each. Human Pathology 18:441-450, 1987.

94. Isner, J.M., Estes, N.A.M., Thompson, P.D., Nordin, M.R., Subramanian, R., Miller, G., Katsas, G., Sweeney, K., Sturner, W.Q.: Acute cardiac events temporally related to cocaine abuse. New England Journal of Medicine 315:1438-1443, 1986.

95. Clarke, R.H., Isner, J.M., Donaldson, R.F., Jones, G.: Gas chromatographic-light microscopic correlative analysis of excimer laser photoablation of cardiovascular tissues. Evidence for a thermal mechanism. Circulation Research 60:429-439, 1987.

Publications, cont'd

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3. Ventricular tachycardia, #4,985,028 granted on 1/15/91
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5. Restenosis, 5,053,033 granted 10/1/91
6. Ventricular tachycardia, #5,104,393 granted on 4/14/92
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Arterial Gene Transfer for Therapeutic Angiogenesis in Patients with Peripheral Artery Disease: Early Clinical Results

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Running head: Vascular gene therapy

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ABSTRACT

Background: Clinical investigation of arterial gene therapy for therapeutic angiogenesis was performed in 7 patients with critical limb ischemia, using a dose-escalating, open label, unblinded protocol. The primary objective of this Phase 1 study was to document the safety of $phVEGF_{165}$ arterial gene therapy for therapeutic angiogenesis. The secondary objective was to determine the bioactivity of arterial gene therapy using $phVEGF_{165}$ to relieve rest pain and/or heal ischemic ulcers of the lower extremities in patients with peripheral artery disease.

Methods and Results: Percutaneous arterial gene transfer was performed in 4 patients with ischemic ulcers and lower extremity rest pain, and 3 patients with rest pain alone. A hydrogel-coated balloon angioplasty catheter was used to perform percutaneous arterial gene transfer of $phVEGF_{165}$, a eucaryotic expression plasmid encoding for the 165-amino acid isoform of vascular endothelial growth factor (VEGF), to a lower extremity arterial site. Patients received 100 μg (n=1), 500 μg (n=1), or 1000 μg (n=5) of naked plasmid DNA without adjunctive vectors.

The 3 patients who presented with rest pain alone, each of whom received 1000 μg of $phVEGF_{165}$, experienced resolution of rest pain and remain free of rest pain three-months post-gene transfer. In all 3 patients, magnetic resonance angiography (MRA) documented improved perfusion of the infrapopliteal circulation; a corresponding improvement in total limb blood flow

was documented using an intravascular Doppler wire; and acceleration of contrast media transit time from the site of injection (common femoral artery) to the ankle was observed.

Among the 4 patients who presented with ischemic ulcers, ulcer size stabilized transiently in one patient who received 1000 μg, but continued to progress in the other three patients treated with 100 μg, 500 μg, and 1000 μg respectively.

Follow-up intravascular ultrasound examination disclosed no evidence of new atherosclerotic plaque at the site of gene transfer in any patient. Likewise, no patient experienced new-onset peripheral edema, ophthalmologic complications, or other systemic toxicity attributable to phVEGF$_{165}$ arterial gene transfer.

Conclusion: Arterial gene transfer of naked DNA encoding for vascular endothelial growth factor can be performed safely using percutaneous catheter delivery. In 3 patients, a reduction in rest pain associated with increased blood flow demonstrated by MRA, intra-vascular Doppler measurement, and contrast angiography suggests that this strategy has potential bioactivity as well. Larger doses of naked plasmid DNA and/or adjunctive vectors may be useful to augment the bioactivity of gene therapy for therapeutic angiogenesis.

Key words: gene therapy, angiogenesis, peripheral vascular disease, magnetic resonance angiography (MRA), vascular endothelial growth factor (VEGF), ulcer

INTRODUCTION

The prognosis for patients with chronic critical leg ischemia, i.e. rest pain and/or established lesions which jeopardize the integrity of the lower limbs, is often poor. Psychological testing of such patients has typically disclosed quality-of-life indices similar to those of patients with cancer in critical or even terminal phases of their illness [1]. It has been estimated that 150,000 in toto [2] require lower limb amputations for ischemic disease in the United States per year. Their prognosis after amputation is even worse [3] : the perioperative mortality for below-knee amputation in most series is 5-10% and for above-knee amputation 15-20%. Even when they survive, nearly 40% will have died within two years of their first major amputation; a major amputation is required in 30% of cases; and full mobility is achieved in only 50% of below-knee and 25% of above-knee amputees.

These grim statistics are compounded by the lack of efficacious drug therapy. As concluded in the Consensus Document of the European Working Group on Critical Leg Ischemia [3],"...there presently is inadequate evidence from published studies to support the routine use of primary pharmacological treatment in patients with critical leg ischemia..." Evidence for the utility of medical therapy in the treatment of claudication is no better [4,5]. Consequently, the need for alternative treatment strategies in such patients is compelling.

The therapeutic implications of angiogenic growth factors were identified by the pioneering work of Folkman and colleagues over two decades ago [6-8]. More recent investigations have established the

feasibility of using recombinant formulations of such angiogenic growth factors to expedite and/or augment collateral artery development in animal models of myocardial and hindlimb ischemia [9-15]. This novel strategy for the treatment of vascular insufficiency has been termed "therapeutic angiogenesis" [16].

Among the various growth factors which have been shown to promote angiogenesis [17,18], vascular endothelial growth factor (VEGF) 19, also known as vascular permeability factor (VPF) [20] and vasculotropin (VAS) [21], is an endothelial-cell specific mitogen. Because endothelial cells represent the critical cell type responsible for new vessel formation [22-24], and because smooth muscle cells - one of the critical cell types responsible for the development of certain vascular lesions [25-27] - would not be directly acitvated, cell-type specificity has been considered to represent an important advantage of VEGF for therapeutic angiogenesis.

VEGF is further distinguished from other angiogenic cytokines by the fact that the first exon of the VEGF gene includes a secretory signal sequence which permits the protein to be naturally secreted from intact cells [28]. Previous studies from our laboratory [29,30] had shown that arterial gene transfer of cDNA encoding for a secreted protein could yield meaningful biological outcomes despite a low transfection efficiency. We therefore performed pre-clinical animal studies to establish the feasibility of site-specific gene transfer of phVEGF$_{165}$, encoding the 165-amino acid isoform of VEGF, applied to the hydrogel polymer coating of an angioplasty balloon [31], and delivered percutaneously to the iliac artery of rabbits in which the femoral artery had been excised to cause unilateral hindlimb ischemia [32]. Analysis of

the transfected internal iliac arteries using reverse transcription-polymerase chain reaction (RT-PCR) confirmed reproducible gene expression at the mRNA level for up to 21 days post-gene transfer [33]. Augmented development of collateral vessels was documented by serial angiograms in vivo, and increased capillary density at necropsy [34]. Consequent amelioration of the hemodynamic deficit in the ischemic limb was documented by improvement in the calf blood pressure ratio (ischemic/normal limb), as well as increased resting and maximum vasodilator-induced blood flow [35] in the VEGF-transfected animals versus controls transfected with a reporter gene. These findings formed the basis for the current clinical investigation.

METHODS

Patients. The criteria for patient inclusion, as well as all other procedural aspects of this clinical investigation, were approved by the Human Institutional Review Board (HIRB) and Institutional Biosafety Committee (IBC) of St. Elizabeth's Medical Center, the Recombinant DNA Advisory Committee (RAC) of the National Institutes of Health, and the U.S. Food and Drug Adminsitration (FDA), and have been described previously. Briefly, men and non-pregnant women, 40 years or older, are considered candidates for this trial: a) if they have narcotic-dependent rest pain of 4 weeks duration or greater; and/or non-healing ulcers which have been present for 8 weeks or greater without response to conservative measures; and are not satisfactory candidates, by reason of anatomy and/or co-morbid illnesses, for non-surgical or surgical revascularization. Furthermore, resting ankle-brachial index (ABI) in the affected limb

must be < 0.6 on two consecutive examinations performed at least 1 week apart. For patients with non-compressible vessels, the toe: brachial index (TBI) must be < 0.6. Finally, diagnostic angiography must unequivocally demonstrate occlusive arterial disease in the ischemic limb.

Patients are excluded if they fail to meet the aforementioned criteria and/or in addition have a history of peripheral vessel angioplasty and/or surgery within the prior two months; previous or current evidence of a neoplasm [36]; clinical evidence of Type I diabetes mellitus, diabetic retinopathy, and/or other ophthalmologic complications of diabetes [37,38]; or a concomitant disease process with a life expectancy of < 1year.

Based upon these selection criteria, a total of 157 patients were evaluated up to and through recruitment of the seventh patient described in this report. Patients were excluded because they lacked signs and/or symptoms of critical limb ischemia (n=74); were judged to be satisfactory candidates for non-surgical (15) or surgical (5) revascularization; had a history and/or evidence of a neoplasm (n=24); had Type I diabetes and/or secondary ophthalmologic complications (n=5); and could not or chose not to comply with the protocol (n=27).

Certain clinical features of the 7 treated patients described in this report are summarized in Table 1.

Plasmid DNA. *Plasmid structure.* The cDNA used in this protocol encodes the 165-amino acid isoform of VEGF and has been described previously [28,39]. The eucaryotic expression vector into which the VEGF cDNA has been inserted, phVEGF$_{165}$, utilizes the 763bp cytomegalovirus (CMV) promoter/enhancer to drive VEGF expression. Downstream from the VEGF cDNA is the SV40 polyadenylation sequence. These fragments occur in the PUC118 vector which includes an *E. coli* origin of replication and the β-lactamase gene for ampicillin resistance. To confirm identity, the entire plasmid sequence was determined by the cycle sequencing method using fluorescent dideoxy terminator nucleotides with an Applied Biosystem 373A Automated sequencer (Foster City, CA). Sequence of the VEGF coding region was determined on both strands and it was in 100% agreement with the predicted sequence.

Plasmid preparation. The plasmid DNA administered to patients in this trial is prepared in an enclosed wing of the Medical Center's Human Gene Therapy Laboratory committed exclusively to the production of phVEGF$_{165}$. DNA was prepared from cultures of phVEGF$_{165}$-transformed *E.coli*. Briefly, plasmid batches were routinely grown in LB media supplemented with ampicillin from a master glycerol stock of transformed *E. coli*..Cells were harvested at a density of 1.0 to 1.5 (A600 units/ml). Plasmid DNA was purified with a Qiagen-tip 2500 column according to the directions of the manufacturer (Qiagen, Chatsworth, CA). Following elution from the Qiagen column, the plasmid DNA was precipitated with isopropanol, and the DNA pellet was washed with 70% ethanol and dried on a Speed Vac. The purified

plasmid was reconstituted in sterile saline and stored in vials. These plasmid batches were pooled prior to further analyses, and, ultimately, administration to patients.

Plasmid monitoring. Batches of plasmid were analyzed for potential protein, endotoxin, microbial, nucleic acid and alcohol contaminations. To determine apparent protein contamination, aliquots of plasmid DNA were evaluated by determining the ultraviolet absorbance at wavelengths of 260 and 280 nm on a Hewlett-Packard 8452A diode array Spectrophotometer (Waldbronn, Germany). An $A_{260/280}$ ratio between 1.75 and 1.85 was required for lot approval. The Limulus Amebocyte Lysate (LAL) gel-clot method, performed by BioWhittaker (Walkersville, MD), was used to quantitatively assess bacterial endotoxin levels. Bacterial endotoxin levels of less than 300 endotoxin units/mg of phVEGF$_{165}$ was required. To ensure that the Qiagen plasmid purification was sufficient to eliminate contamination with adventitious agents such as bacteria, fungi, or mycoplasma, aliquots of the plasmid DNA were evaluated in accordance with CFR 610.12 by the Clinical Microbiology Laboratory at St. Elizabeth's Medical Center. An aliquot of each plasmid batch representing 1 μg of unrestricted phVEGF$_{165}$ plasmid was also be analyzed by ethidium bromide staining following electrophoresis on a 0.7% agarose gel. Greater than 90% of the nucleic acid was required to be in the closed, circular supercoiled form. The level of contaminating genomic *E. coli* DNA in the phVEGF$_{165}$ plasmid preparation was detected by Southern (slot) blot. Total genomic DNA (50 to 150 kbp), radiolabeled by random priming, was used as a probe against the phVEGF$_{165}$ plasmid DNA and known amounts of genomic DNA. Less than 50 ug of genomic

DNA per mg of plasmid was required for lot release. Aliquots of plasmid were also analyzed for low molecular weight RNA species by electrophoresis on a native 0.7% agarose gel followed by staining with ethidium bromide. Lack of detectable RNA in 1 ug aliquot of phVEGF$_{165}$ plasmid was required. Plasmid batches were also analyzed for contaminating isopropanol and ethanol by gas chromatography by the Institute Fresenius GmbH (Hilden,Germany). All batches tested had alcohol levels below the limits of detection.

To confirm the identity of the plasmid following its preparation, aliquots were digested with appropriate restriction enzymes to confirm that the pattern of generated fragments conformed to the predicted digestion pattern. Diagnostic restriction enzyme digests were performed with DraI, PvuII, and EcoRI (separately). Finally the VEGF-coding region was resequenced using an aliquot from each pooled plasmid batch.

Plasmid reconstitution. Plasmid DNA was reconstituted on the day of arterial gene transfer. The concentration and final volume employed varied as a function of dose of plasmid DNA as follows: 100 μg plasmid DNA, prepared as 0.87 μg/μl/ in 115 μl for Pt. 1; 500 μg, at a concentration of 7.4 μg/μl in 67.6 μl for Pt. 2; and 1000 μg, at concentrations of 6.0 to 9.0 μg/μl, in 167 to 111.1 μl respectively for Pts. 3 through 7 (Table 2).

Angiography, ultrasound,and Doppler examinations. After access had been established to the common femoral artery, baseline digital subtraction angiograms were recorded using undiluted non-ionic contrast media (Isovue-370, Squibb Diagnostics, New Brunswick, NJ) delivered antegrade and selectively into the ischemic limb. Intravascular ultrasound (IVUS) examination was then performed as described previously [40] by advancing over a 0.018 in. guidewire, a 3.5 F, 20 MHz catheter (Sonocath, Boston Scientific, Natick, MA) interfaced to an imaging console adapted for 20 MHz operation and 360° scans (Hewlett-Packard, Andover, MA). Luminal dimensions were measured in reference to a stationary radiographic ruler positioned parallel to the limb bones. Maximum lumen diameter (in mm) was taken as the length of the longest line that could be constructed between the inner edge of the luminal-intimal interfaces from contralateral borders and passing through the center of the lumen; lumen area (in mm^2) was measured as the area enclosed by the leading edge of the luminal-intimal border [41]. Intravascular determination of flow velocity was measured using a 0.018 in. guidewire with a Doppler crystal mounted at its distal tip (FloWire, Cardiometrics, Mountain View, CA) [42]; resting and maximum flow were measured respectively pre- and post-300 µg of nitroglycerin administered intra-arterially via the side-arm of the introducer sheath. Doppler-derived flow was calculated as $Q_D=(\pi d^2/4)$ (0.5 X APV), where Q_D is Doppler-derived time average flow (ml/min), d is vessel diameter, and APV is time average of the spectral peak velocity [43]. The mean velocity was estimated as 0.5 X APV by assuming a time-averaged parabolic velocity profile across the vessel. The Doppler-derived flow calculated in this fashion has been

shown to correlate with flow measurements determined by electromagnetic flowmeters both in vitro and in vivo [43].

Percutaneous arterial gene transfer. Arterial gene transfer was accomplished using a hydrogel-coated balloon angioplasty catheter (Boston Scientific) described previously [31]; the diameter of the balloon was selected to approximate a 1:1 ratio with regard to the arterial diameter at the site selected for gene transfer. The balloon catheter was first advanced, ex vivo, through a protective sheath, the diameter of which was selected to closely approximate that of the balloon catheter (Table 2); the purpose of the sheath was to minimize contact between the coated hydrogel balloon and flowing blood, following introduction of the catheter into the lower extremity circulation. A sterile pipette was used to apply the solution of plamid DNA to the external hydrogel coat of the balloon inflated to 3 atm. Following each application, the plasmid DNA was blown dry using room-temperature air; this procedure was repeated until the total volume of plasmid DNA had been applied to the balloon. The balloon was then deflated, retracted into the sheath, and reinflated to 3 atm. The balloon catheter and sheath were then advanced via a standard guidewire under fluoroscopic guidance to the site of gene transfer; the balloon was then deflated, the sheath retracted, and the balloon reinflated at nominal inflation pressures for 4 to 5 min. The balloon was then deflated, all catheters and wires removed, and a final angiogram recorded to ensure satisfactory patency of the gene transfer site.

Pre- and post-gene therapy assessment. *History.* Patients were interrogated regarding the presence or absence of rest pain, as well as claudication. The generic name and total daily dose of all analgesic or other drugs was recorded. The dates of any required surgery for extensive debridement, skin grafting or limb amputation was recorded.

Physical examination. A general as well as specific vascular examination was performed. Ischemic ulcer(s) were characterized as wet or dry, identified as to site, and documented by a gross photograph. The size of each ulcer was measured according to the longest length and the longest perpendicular width. The depth of each ulcer was graded as 1 (superficial), 2 (involvement of subcutaneous tissue), 3 (exposure of tendon or bone), or 4 (necrosis of tendon or bone). Limb circumference was measured at the calf and ankle levels pre- and at regular intervals post-gene transfer.

Ankle-brachial index (ABI). The ABI was obtained with the patient supine after resting supine for ≥20 min. A continuous wave Doppler ultrasonic device (Parks Medical Electronics, Aloha, OR) was used to detect the arterial signal, and standard pneumatic cuffs were used to measure ankle systolic pressure from both the dorsalis pedis and posterior tibial arteries of each leg, as well as the brachial pressure in each arm.

Magnetic resonance angiography (MRA). Six patients underwent MRA pre- and post-gene therapy; Pt. 4 (Table 1) did not undergo MRA examinations due to the presence of a permanent

pacemaker. Imaging was performed using a 1.0 Tesla magnetic resonance imaging system (Impact, Siemens, Erlangen, Germany). A multisection 2D time of flight [44] gradient echo sequence was used without intravenous contrast media. A series of acquisitons was performed from the pelvis to the ankle. A body coil was used for the larger portions of the lower extremity, typically from the pelvis to the adductor canal region. A circularly polarized array coil was used when possible, typically from the knee to the level of the malleoli. A maximal intensity projection algorithm was used for image reconstruction at 30 degree increments.

The studies were all interpreted by one radiologist (RB) who was blinded to the date of gene transfer. For each patient, comparison was made between studies using identical fields of view. Images were evaluated in both axial and longitudinal formats for intensity of vessel flow, including corresponding vessel diameter.

Angiography, intravascular ultrasound and intravascular Doppler flow examination. For angiography, a catheter or introducer sheath with dimensions identical to that used to perform the baseline examination was also used for the follow-up study. Likewise, the volume of undiluted contrast media, rate of injection, imaging obliquity, optical magnification, and field of view were all identical to those employed initially . Multiple levels from the groin to the ankle were recorded using digital acquisition and hard-copied in a 14X14 format for further analysis. For IVUS examination, a radiographic ruler, bony landmarks, and vessel side-branches visualized on the image recorded during gene transfer from the

inflated balloon were used to ensure accurate positioning for follow-up measurements of the gene delivery site. Likewise, for follow-up intravascular Doppler blood flow studies, the distal tip of the Doppler wire was positioned using a hard-copy film recorded during the initial flow-wire recording

RESULTS

Clinical course. The first seven patients to receive phVEGF165 arterial gene transfer included 5 men and 2 women ranging in age from 54 to 92 years (m+SEM=71.9±12.6) (Table 1).

Four patients presented with ischemic ulcers of the foot and/or toes. In these four patients, the size of the ulcers at the time of presentation ranged from 1 cm. X 1 cm., to 7 cm. X 3 cm; the depth ranged from 2 (2 patients) to 3 (2 patients) cm. During the time required to complete screen tests prescribed by the protocol prior to gene therapy, tissue loss continued to progress in all 4 patients, despite optimized foot care. By the time of gene transfer, the ulcers ranged in size from 1cm.X 1 cm. to 5 cm.X 8 cm.

In one of the two patients with an ulcer (Pt. #3, Table 1) who received 1000 μg of phVEGF$_{165}$, the size and depth of the ulcer appeared to stabilize for a period of approximately 2 months following arterial gene transfer; the progressive increase in the size of this ulcer pre-gene therapy versus the apparent plateau observed for 2 months post-gene therapy. During these 2 months, the patient experienced a reduction in rest pain, sufficient to allow him to proceed with a two-week trip to Germany for a reunion of

former U.S. and German generals who fought in the Battle of the Bulge. Near the end of this trip, which involved extensive walking across previous battlefields, the patient experienced an increase in rest pain, and the ulceration of the great toe became gangrenous. Subsequently, the patient was referred back to his physician for amputation of his left great toe and distal bypass surgery using a composite graft. Two weeks following surgery, the patient returned for a transmetatarsal amputation

Three patients presented with claudication and rest pain unassociated with loss of tissue integrity. Rest pain in each of these patients was manifested principally by nocturnal episodes of forefoot pain waking the patients from sleep; the pain was typically relieved by placing the affected limb in a dependent position. At three months post-gene therapy, all three patients remain free of rest pain. Prior to gene therapy, all three patients had also complained of claudication at less than 100 yards on a level surface. At three-month follow-up, Pt. 5 was walking $\geq$ 0.5 miles/day without pain; in Pts. 6 and 7 the extent of pain-free walking was unchanged.

Non-invasive testing. *Calf/ankle girth.* In 2 patients, grossly apparent ankle edema (2+/4+ in Pt.1, 3+/4+ in Pt. 4) was present prior to gene therapy and persisted to a variable degree post-gene therapy. In the remaining 5 patients, measurement of calf and ankle circumference, as well as non-quantitative gross inspection, disclosed no new-onset edema post-gene therapy.

Ankle-brachial index (ABI). The ABI measured prior to gene therapy ranged from 0 to 0.47 (0.31±0.15). Compared to the ABI measured prior to gene therapy, the mean value of measurements recorded for each patient at weekly intervals up to three months post-gene therapy did not improve by >0.1, the increment suggested to represent a significant change following angioplasty or reconstructive surgery [45].

Magnetic resonance angiography (MRA). One patient (Pt. 4) with a permanent cardiac pacemaker was not studied by MRA. In 3 patients (Pts. 1-3), no significant change was apparent on serial assessment of MRA scans. In the last 3 patients to receive the 1000 μg dose, however, MRA performed subsequent to gene transfer demonstrated improved perfusion of the infrapopliteal circulation. In Pt. 5 (Fig. 1), optimization of signal intensity was noted by 6 weeks, involving a large corkscrew-appearing collateral and the peroneal artery subserved by this collateral artery. In Pt. 6 (Fig. 2), improvement in flow, inferred from an increase in signal intensity in the posterior tibial and peroneal arteries, was observed by 3 weeks follow-up. In Pt. 7 (Fig. 3), improved flow involving all three major infrapopliteal arteries, but most prominent in the peroneal and posterior tibial, was optimal by 4 weeks post-gene transfer; geniculate collateral vessels reconstituting the distal popliteal artery also showed increased flow in comparison to the MRA recorded pre-gene transfer.

Invasive testing. *Angiography.* Baseline angiograms were compared to angiograms recorded at last follow-up (4 weeks in Pts. 1and 2; 1 week in Pt. 4; and 12 weeks in Pts. 3, 5,6, and 7) with regard to patency of the gene transfer site; new collateral arteries; and contrast transit time (common femoral artery to pedal arteries). As described above, gene transfer in Pt. 4, performed immediately upstream from the site of total occlusion of the tibio-peroneal trunk extended the original occlusion retrograde to involve the site of gene transfer; no side-branches or pre-exisiting collaterals were compromised. This was the only case in which gene transfer was performed in an occluded vessel, and perhaps as a related consequence, was the only patient in whom follow-up angiography disclosed compromised patency at the transfection site. In all other cases, angiography disclosed no visible alteration at the site of gene transfer.

Inspection of comparable views obtained at identical obliquities, using identical preparations, concentrations, and volumes of contrast media failed to identify angiographically apparent, discrete new collateral vessels in any of the seven patients. "Blushes" of contrast opacification without discrete neovascularity were disregarded for the purpose of this analysis.

The most noteworthy change apparent upon inspection of the follow-up angiograms was acceleration of contrast transit time in Pts. 5,6 and 7. Using identical conditions (including catheter/sheath size and position; contrast preparation, concentration, and volume; and field of view), the time required for arrival of contrast media in the pedal vessels following injection into the common femoral artery for Pt. 6

was 40 sec prior to gene therapy, and 9 sec at last follow-up. For Pt. 5, contrast transit time was reduced by 13 sec, and for Pt.7, by 5 sec. Contrast transit time was not different at last follow-up for any of the remaining 4 patients.

Intravascular ultrasound (IVUS). IVUS examination performed in preparation for gene transfer identified accessible arterial segments for arterial gene transfer that were free of atherosclerotic narrowing in 5/7 patients; the arterial wall at the site of gene transfer in these 5 patients had a clearly recognizable 3-layer appearance, indicating minimal to absent intimal thickening [46]. In 2 patients (Pts. 2 and 3), no site could be identified in either the SFA or deep femoral artery that was similarly pristine; the site least narrowed by atheroslcerotic plaque was therefore selected for arterial gene transfer.

IVUS inspection of the site of arterial gene transfer was repeated in 6/7 patients at 4 weeks (Pts. 1,2) or 12 weeks (Pts. 3, 5,6, and 7) post-gene transfer. In Pt. 4, as described above, gene transfer performed immediately proximal to the site of occlusion in the popliteal artery resulted in retrograde propagation of the original occlusion for approximately 2 cm, occluding the gene transfer site. In the remaining six patients, arterial gene transfer to a normal (four patients) or mildly atherosclerotic (two patients) artery did not compromise vessel patency; specifically, subsequent IVUS examinations disclosed no new intimal thickening up to three months post-gene transfer (Figs. 4,5).

Intravascular Doppler flow analysis. Intra-arterial blood flow was measured in the ischemic limb at the time of three-month follow-up angiography in Pts. 3, 5, 6, and 7. In comparison to flow

measured immediately prior to arterial gene transfer, no improvement in flow was observed in Pt. 3. In the remaining 3 patients, however, including all 3 patients (Pts. 5, 6, and 7) in whom evidence of clinical improvement was observed, improved flow was documented at three months post-gene transfer (Fig. 6). The improvement in flow included both flow measured at rest (132.3 to188.5% of baseline), as well as that recorded 30 to 90 secs following administration of the endothelium-independent vasodilator, nitroglycerin (120.0 to 158.6% of baseline).

Additional testing. *Ophthalmologic examination.* In no patient were changes noted in funduscopic examination performed at three-months follow-up.

Complications. One patient (Pt. 3) developed a pseudoaneurysm at the site of antegrade cannulation for arterial gene transfer. This was treated successfully by ultrasound-guided compression and resolved without sequelae.

DISCUSSION

Beginning with the reports of Nabel et al [47,48], work from several laboratories [49-57] convincingly demonstrated the feasibility of arterial gene transfer. Our preliminary experience with arterial gene transfer in the treatment of peripheral vascular disease extends these previous studies performed in live animals to human subjects. No adverse consequences attributable to the recombinant protein encoded by phVEGF$_{165}$ were observed. VEGF increases vascular permeability when assessed by the Miles assay [58]

(accounting for its alternative designation, vascular permeability factor or VPF [20,59,60]), and has been implicated in the development of proliferative retinopathy [37,38]. Among the current series of patients, however, serial measurement of ankle and calf circumference disclosed no evidence of new peripheral edema. Likewise, serial ophthalmologic examinations disclosed no pathologic eyeground changes. The absence of these complications is consistent with site-specific activity observed in pre-clinical animal studies of VEGF, administered either as plasmid DNA [33] or the recombinant protein [61]; such site specific angiogenesis appears to be mediated by paracrine induction of VEGF receptors in endothelial cells exposed to factors secreted by hypoxic myocytes [62].

From a technical standpoint, one case of femoral artery pseudoaneurysm, cited previously as the most common access-site of complication among patients undergoing percutaneous interventions, [63] was treated conservatively and resolved without sequelae. In a second patient, gene transfer performed immediately proximal to the site of occlusion in the popliteal artery resulted in retrograde propagation of the original occlusion for approximately 2 cm, occluding the gene transfer site. This was the only patient in whom gene transfer was performed immediately proximal to a pre-existing total occlusion; this particular site was selected in an attempt to deliver the transgene as far distally in the limb, i.e. as close to the ischemic forefoot ulcer, as possible. In the remaining six patients, arterial gene transfer to a normal (four patients) or mildly atherosclerotic (two patients) artery did not compromise vessel patency; specifically, IVUS examinations disclosed no new intimal thickening up to three months post-gene transfer. It is

indeed likely that phVEGF$_{165}$ gene transfer accelerates re-endothelialization, and thereby obviates luminal compromise of the transfected segment [64].

Because the current investigation is a Phase 1 trial, evidence of bioactivity was considered a secondary objective. Furthermore, a dose-escalating strategy was mandated for this trial due to the fact that VEGF, either as a recombinant protein or otherwise, had not been previously administered to human subjects. In contrast to the dose employed in pre-clinical animal studies (500 μg, or 0.114 mg/kg for 3.5 kg rabbits), the dose of plasmid DNA for the first two patients was limited to 100 and 500 μg (0.001 and 0.007 mg/kg) respectively. The subsequent five patients were approved for 1000 μg (0.014 mg/kg). At what may still be considered to be a relatively low dose (the dose will ultimately escalate to 4000 μg for the final seven patients of the total 22 approved for this Phase 1 trial), evidence of bioactivity was nevertheless observed. In three of the four patients treated with 1000 μg of plasmid DNA in whom the gene transfer site remained patent, evidence of augmented flow to the distal portion of the ischemic limb was documented by three independent modalities. In these three patients, intravascular Doppler analysis disclosed an increase in both resting flow (132.3 to 188.5% of baseline), and maximum flow (120.0 to 158.6%) provoked by intra-arterial nitroglycerin.; these results compare favorably with the mean increase in rest (140%) and maximum (173%) flow documented in the rabbit model of limb ischemia 30 days following administration of VEGF$_{165}$ recombinant protein [65]. Contrast-negative MRA graphically confirmed the increased infrapopliteal blood flow in these three patients, and contrast angiography

documented accelerated delivery of contrast media from the common femoral artery to the pedal arch vessels in these three patients as well. Moreover, these latter three patients, each of whom presented with several (6 to 17) months of ischemic rest pain, remain free of rest pain at three-month follow-up. In the fourth patient, in whom rest pain was associated with aggressive growth in the size of an ischemic ulcer during the three months prior to gene therapy, further extension of the ulcer was blunted for two months post-gene therapy.

Recently reported clincial trials of human gene therapy for cystic fibrosis [66] and Duchenne's muscular dystrophy [67] yielded somewhat disappointing results, perhaps in part related to the challenge of expressing the gene product - which in both of these cases remains intracellular - among a large proportion of airway epithelia or skeletal myocytes respectively. In the current protocol, the requirement for a higher transfection efficiency may be obviated by the fact that VEGF protein includes a leader sequence which permits active secretion from intact cells; thus, even if VEGF gene expression is limited to a small number of cells, the paracrine effects of the secreted gene product may be sufficient to achieve a meaningful biologic effect. The question, however, as to whether naked plasmid [49,68-72] will suffice, or whether, in spite of the secreted feature of the gene product, the magnitude of gene expression required will demand the use of adjunctive, including viral, vectors [51-57,73-75] remains to be addressed. If naked plasmid DNA alone is to be used, then the optimal dose of plasmid DNA remains to be established. Other critical issues which remain to be clarified include the optimal frequency of administration; if the gene product is limited

to a 30-day window - as suggested by preclinical studies [33] - then the time interval required for full maturation of a lengthy collateral network might benefit from repeated administration, three weeks, for example, after the first dose. The extent to which a favorable response is affected by the proximity of the site of gene transfer to the ischemic focus in the affected limb also remains uncertain.

The more global issue regarding the relative merits of gene therapy versus administration of the recombinant protein for achieving therapeutic angiogenesis also remains uncertain. The non-availability of VEGF recombinant protein for human subjects makes this currently a moot point. Should the protein be made available for human testing, it will be intriguing to see whether the slow-release depot aspect of gene therapy [76], administered in a site-specific fashion and targeted to local pathology, will yield outcomes which are superior to that which can be achieved with bolus and/or continuous administration of the protein.

Certain limitations of the current study must be explicitly underscored. With regard to safety, these findings are preliminary and do not establish the long-term safety of VEGF, administered either as a gene or gene product. Likewise, the preliminary nature of the results dictates that evidence of bioactivity, while encouraging, must be viewed cautiously. This is particularly so given that this first phase of clinical investigation was non-randomized. While consideration was given to the issue of a control group, the HIRB, RAC, and FDA concurred that there was limited justification for undertaking catheter manipulation in patients with marginal limb perfusion and extensive atherosclerosis solely for the purpose of performing

a sham transfection. For the patient undergoing gene transfer, the procedural risks were offset by the potential for relief from unremitting rest pain or healing of refractory ulcers; for the patient undergoing a sham transfection, the risks would not be offset by any potential benefit. To minimize the likelihood of spontaneous improvement in either rest pain and/or the appearance of an established ulcer, inclusion criteria required, in the case of rest pain alone, a minimum duration of four weeks of rest pain with dependence on narcotics without improvement; and, in the case of non-healing ulcerations, a minimum of four weeks of conservative therapy without evidence of healing. While rest pain and/or ulcerations of this nature may precipitously deteriorate, the potential for spontaneous improvement under these circumstances is remote [3]. The short-term nature of the follow-up obtained to date also leaves undetermined the durability of apparent clinical improvement observed in selected patients.

The precise mechanism responsible for the salutary effects observed in patients who received the 1000 µg dose of DNA remains uncertain. What we have observed, by three independent examinations, is evidence of increased flow to the distal extremities, specifically distal to the preexisting occluded vessels. This was most graphically illustrated by MRA performed pre- and post-gene therapy. In Pt. 5, for example, striking reconstitution of the distal peroneal artery developed in association with a similarly lengthy occlusion of the SFA/popliteal artery. In Pt. 6, flow was improved to both the peroneal and posterior tibial. In Pt. 7, flow appeared substantially increased in the posterior tibial, peroneal, and to a lesser degree, in the anterior tibial - all distal to the SFA/popliteal, which was occluded over its entire

length. In each of these patients, measurement of increased blood flow using an intravascular Doppler wire, and accelerated transit of angiographic contrast media supported the results of MRA.

These findings are consistent with experimental observations described recently by Pearlman et al [14], who used magnetic resonance imaging (MRI) to study the time delay in delivery of contrast media to the collateral-dependent myocardium of pigs in which the circumflex coronary artery was occluded by an ameroid constrictor. Following six weeks of treatment with VEGF (recombinant protein), contrast arrival time in the myocardium subserved by the occluded circumflex was markedly accelerated. Because survival and function of this ischemic myocardial zone is dependent upon collateral flow, the observed improvement in contrast delivery was inferred to represent augmented neovascularity, although direct demonstration of same was not shown. Previous DNA labeling studies in this swine model [77], a similar canine model [78], and the ischemic rabbit hindlimb [79] have established that improvements in flow associated with collateral development are typically associated with proliferation of new vessels <180μ in diameter, including a statistically significant increase in capillary density. We presume that among the three patients described above, flow from the profunda to the infrapopliteal vessels was improved via an augmented network of collaterals. Direct evidence of new blood vessel formation, however, remains pending, either because the size of the new vascular structures is beyond the resolution of conventional angiography, or because of other as yet undisclosed reasons.

We have also considered the alternate possibility that the increase in distal extremity blood flow might be the result of vasodilation. VEGF has been shown in vivo to produce endothelium-dependent hypotension that can be blocked and/or reversed by administration of N^G-monomethyl-L-arginine (L-NMMA), an inhibitor of nitric oxide synthase [80]. Moreover, in vitro studies have demonstrated VEGF-induced relaxation of canine coronary arteries that was abolished by endothelial denudation or pre-treatment with L-NMMA [81], and recent studies in our laboratory have directly documented VEGF-induced release of nitric oxide from isolated rings of enothelium-intact (but not endothelium-denuded) rabbit aorta (R. van der Zee, unpublished data). It is our current feeling, however, that relief of rest pain at 12 weeks accompanied by evidence of augmented flow in the ischemic limb is unlikely to represent a vasodilator effect of VEGF, given that pre-clinical animal studies have consistently demonstrated expiration of gene expression (and, by inference, synthesis of recombinant VEGF protein) between 21 and 30 days post-gene transfer [33].

Finally, it must also be acknowledged that all patients, by virtue of the delivery catheter employed, underwent incidental balloon angioplasty in the course of performing arterial gene transfer. In all cases, however, the site of gene transfer was selected specifically because IVUS examination disclosed no evidence of signficant luminal narrowing; thus any improvement observed cannot be attributed to reduction in luminal narrowing by ballon angioplasty. Thus, balloon dilation alone is unlikely to account for these preliminary observations.

In summary, preliminary data from these first seven patients may be cautiously interpreted to support both the strategy of arterial gene therapy and the concept of therapeutic angiogenesis for the treatment of selected patients with critical limb ischemia. Clearly, however, much additional clinical and basic investigation will be required to establish the extent to which either strategy will be sufficiently potent to constitute a bonafide addition to currently available standard therapies.

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LEGENDS

Fig. 1. Magnetic resonance angiography (MRA, performed without contrast media) pre-and post-gene therapy in Pt. 5. In comparison to the longitudinal reconstruction (top) and tomographic view of the infrapopliteal vessels recorded pre-gene therapy, the study recorded post-gene therapy shows enhanced flow via a tortuous collateral (arrow) to the peroneal (Per) artery distally; flow into the anterior (AT) artery is improved as well.

Fig. 2. MRA pre-and post-gene therapy in Pt. 6: post-gene therapy there is improved flow in the deep femoral (DF) artery, as well as the posterior tibial (PT) and peroneal (Per) arteries.

Fig. 3. MRA pre- and post-gene therapy in Pt. 7: post-gene therapy, markedly improved flow is seen in the posterior tibial (PT) and peroneal (Per) arteries, and, to a lesser degree, in the anterior tibial (AT) artery as well.

Fig. 4. Three-dimensional reconstruction and representative tomographic views of entire length of gene transfer site in Pt. 5. Uniform luminal caliber is preserved, and tomographic views confirm preserved three-layer appearance of arterial wall with no new intimal thickening at any site.

Fig. 5. Hydrogel-balloon catheter employed for arterial gene transfer did not produce intimal thickening. *A-D* are intravascular ultrasound (IVUS) image recorded at last follow-up, up to three months post-gene transfer) in Pts. 5 *(A)*, 1 *(B)*, 7 *(C)* , and 6 *(D)* respectively. In each patient the pristine, three-layer appearance of the normal arterial wall is circumferentially preserved (I=intima; M=media; asterisk indicates IVUS catheter; linear echogenic reflection is guidewire used to advance IVUS catheter). Shown in *E* and *F* are the pre- and post-gene transfer images recorded in Pt. 3, in whom IVUS performed prior to gene transfer dislosed no arterial segment that was free of intimal thickening; the site shown in *E* was the least narrowed site. Follow-up examination at 3 months *(F)* shows no increase in atherosclerotic plaque *(P)*.

Fig. 6. Percent change (pre- versus 3 months post-gene therapy) in resting and maximum (post-nitroglycerin) blood flow in ischemic limb measured by intravascular Doppler flow wire. Results are shown for the 4 patients (Pts. 3, 5, 6, and 7) who completed 3-month follow-up. In Pt. 3, resting and maximum flow were reduced by -6.3% and 25.5% respectively compared to baseline. In Pts. 5,6, and 7, resting flow was increased by 32.3, 88.5, and 59.0%; maximum flow was increased by 20.0, 58.6, and 35.5%.

Table 1. Clinical Features of Patients Treated with phVEGF$_{165}$

Pt	Sex	Age	Cigs	DM	Rutherford Class	ABI	Prev. Rx	Rest Pain +/0	Rest Pain Dur'n.	Meds	Ulcer +/0	Ulcer Dur'n.	Ulcer Loc'n.	Ulcer Size	Depth*	Other	Vascular Occlusion in Affected Limb DF	SFA	Pop AT	PT	Per	BP
1	M	54	+	O	5	.43	Fem-Fem BP; Fem-pop BP*; PTA of Fem-pop BP*	+	7mo	Percocet Fentanyl patch	+	7 mo	foot	8cmx5cm	3	Pitting edema	O	TO	TO	TO	TO	TO
2	M	60	+	+	5	.00	PF, EA, & Fem-pop BP; Revision of BP; PTA of BP x 2*	+	6mo	Percocet	+	6 mo	foot	1cmx1cm to 2cmx2cm	3	O	O	TO	TO	TO	TO	TO
3	M	81	O	O	5	.39	Fem-pop BP; SFA/pop PTA*	+	6mo	E-S Tylenol	+	6 mo	toe	3cmx3cm	2	O	O	TO	TO	TO	TO	TO
4	M	92	O	O	5	.28	Recommended for AKA	+	5mo	Percocet Fentanyl patch Morphine sulfate	+	4 mo	toes	1cmx1cm	2	Pitting edema	O	O	O	TO	TO	NA
5	F	72	O	O	4	.31	Fem-pop BP	+	17mo	Vicoden	O	NA	NA	NA	NA	O	O	TO	TO	O	TO	O
6	F	71	+	O	4	.31	Fem-pop PTA; Fem-per BP; PTA of Fem-per BP; Iliac PTA; Repeat PTA of Fem-per BP	+	6mo	Vicoden Percocet Codeine	O	NA	NA	NA	NA	O	O	TO	TO	TO	O	TO
7	M	73	+	O	4	.47	Fem-pop BP; Fem-AT BP; CABG CEA (bilateral)	+	6mo	Percocet	O	NA	NA	NA	NA	O	O	TO	O	O	TO	TO

*Performed following development of ulcer

* 1=superficial; 2=involves subcutaneous tissue; 3=exposure of tendon or bone; 4=necrosis of tendon or bone

Abbreviations: (+)=yes; (O)=no, ABI=ankle-brachial index; AKA=above-knee amputation; AT=anterior tibial; BP=bypass surgery of lower extremity; CABG=coronary artery bypass graft; CEA=carotid endarterectomy; Cigs=cigarette smoker; DF=-deep femoral; DM=diabetes mellitus; Dur'n.=duration; EA=endarterectomy; F=female; Fem-fem=femoral-femoral; Fem-pop=femoral-popliteal; Loc'n.=location; Meds=analgesic medications; mo=months; NA=not applicable; PF=profundaplasty; Pop=popliteal; Per=peroneal; Prev. Rx=previous angioplasty or surgery in affected limb; Pt.=patient; PT=posterior tibial; R=right; SFA=superficial femoral artery; TO=total occlusion.

Table 2. Catheter Delivery of phVEGF$_{165}$

	Plasmid DNA			PTA Catheter				Access		Gene Tx Site
Pt.	Dose	Conc	Total Vol	BD	BL	Shaft	Sheath	Artery	Intro	
1	100 µg	0.87 µg/ul	115 ul	4.0 mm	2 cm	5F	6F	Graft (C)	8F	DF
2	500 µg	7.4 µg/ul	67.6 ul	6.0 mm	2 cm	5F	6F	CFA (I)	8F	SFA
3	1 mg	9.0 µg/ul	111.1 ul	6.0 mm	2 cm	5F	6F	CFA (I)	8F	SFA
4	1 mg	7.7 µg/ul	129.8 ul	6.0 mm	2 cm	3.8 F	7F*	CFA (I)	8F	POP
5	1 mg	6.0 µg/ul	167 ul	4.5 mm	2 cm	3.8F	5F	CFA (I)	8F	DF
6	1 mg	6.0 µg/ul	167 ul	4.0 mm	4 cm	3.8 F	5F	CFA (I)	8F	DF
7	1 mg	6.0 µg/ul	167 ul	4.0 mm	2 cm	3.8 F	6F	CFA (I)	8F	DF

*Guide catheter (Guidzilla, Schneider, Minneapolis, MN) with internal dimension of .074 mm.

Abbreviations: BD=balloon diameter; BL=balloon length, CFA=common femoral artery; Conc=concentration; C=contralateral; DF=deep femoral; Diam=diamter; F=French size; I=ipsilateral; Intro=introducer sheath; POP=popliteal; Pt.=patient, PTA=percutaneous transluminal angioplasty; SFA=superficial femoral artery; Tx=transfer; Vol=volume

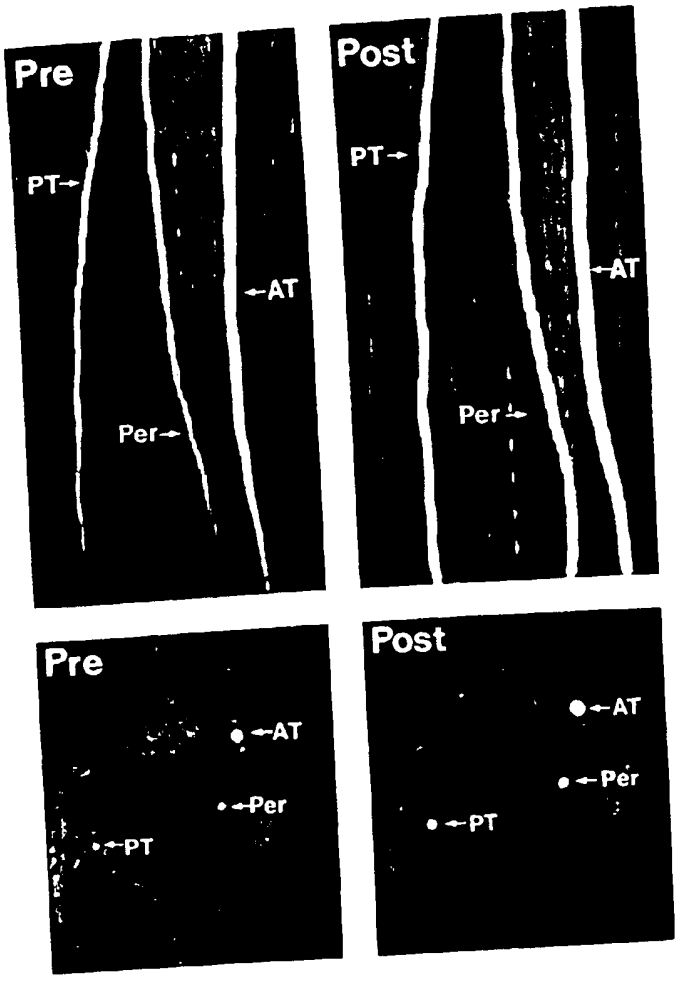

Fig. 1
Vascular gene therapy
Isner et al

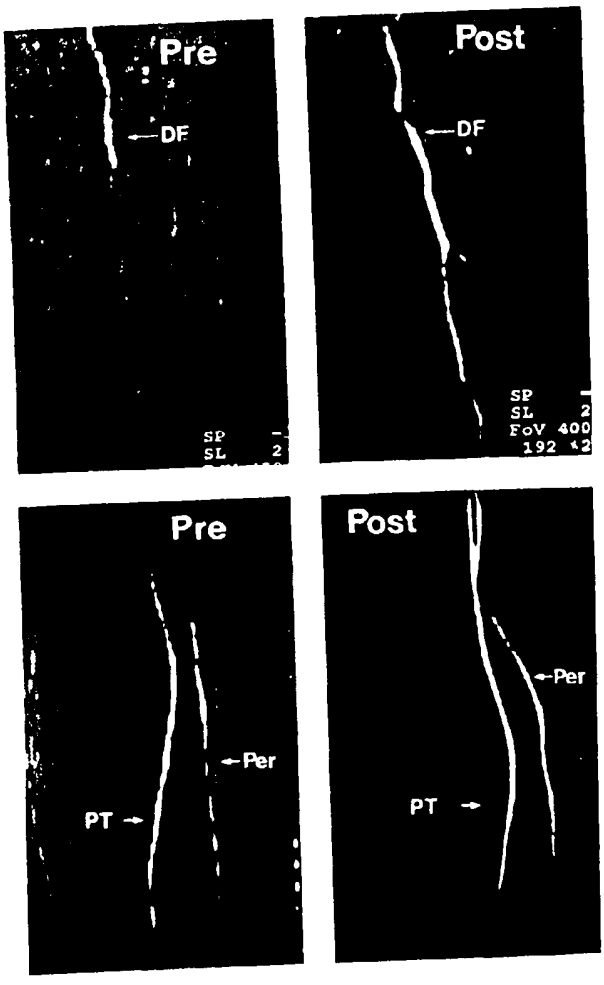

Fig. 2
Vascular gene therapy
Isner et al

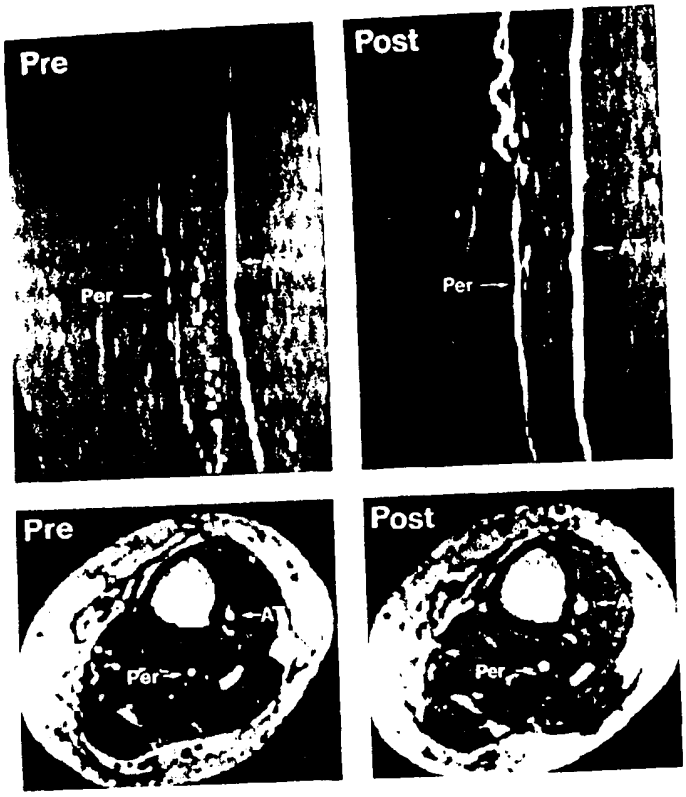

Fig. 3
Vascular gene therapy
Isner et al

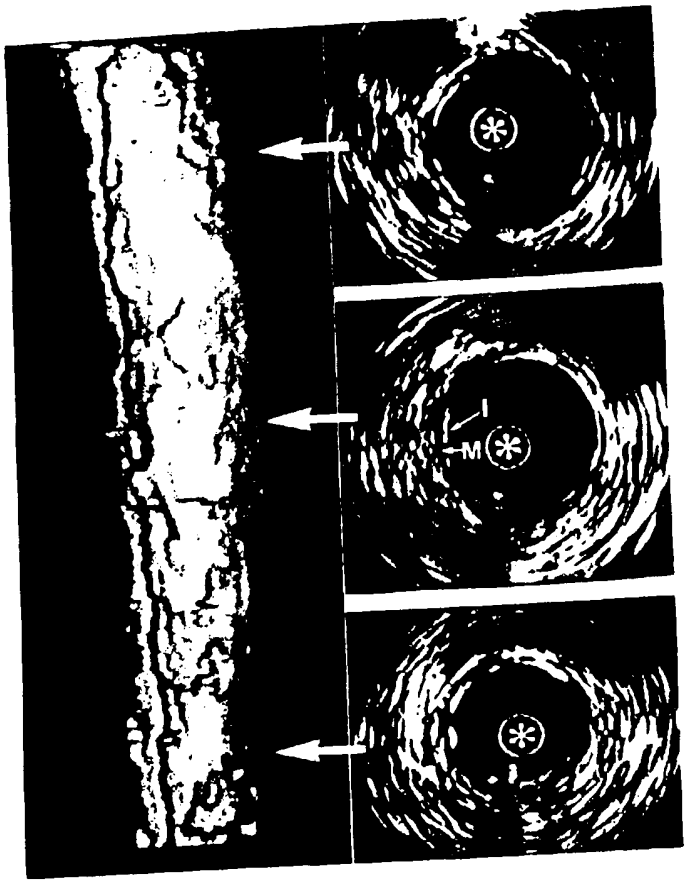

Fig. 4
Vascular gene therapy
Isner et al

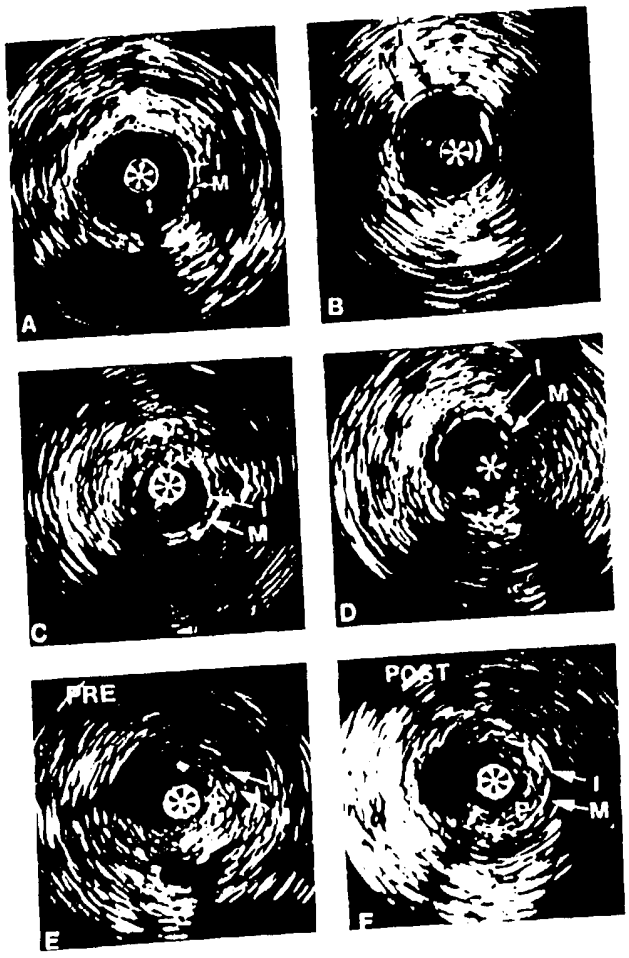

Fig. 5
Vascular gene therapy
Isner et al

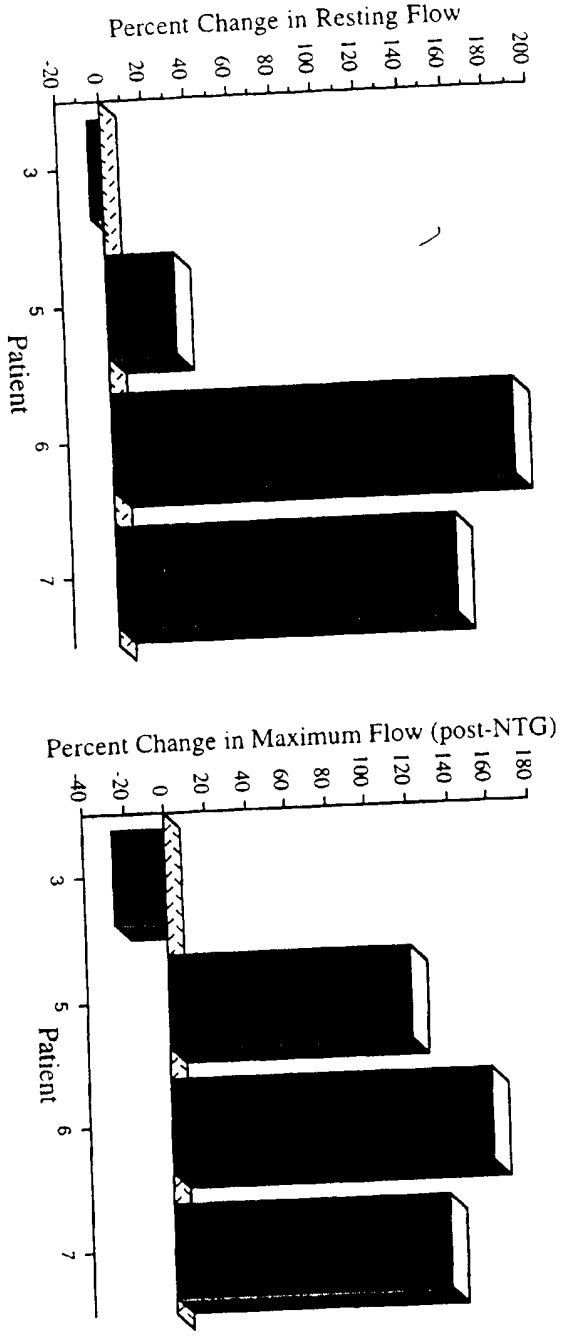

Fig. 6
Vascular gene therapy
Isner et al

#21

Docket No. 44559-FWC

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: Jeffrey M. Isner EXAMINER: Low, C.

SERIAL NO. 08/675,523 GROUP: 1804

FILED: July 3, 1996

FOR: METHOD AND PRODUCTS FOR NUCLEIC ACID DELIVERY

THE HONORABLE COMMISSIONER
OF PATENTS AND TRADEMARKS
WASHINGTON, DC 20231

Sir:

DECLARATION OF DR. JEFFREY M. ISNER
UNDER 37 C.F.R. §132

I, Jeffrey M. Isner, hereby declare:

1. I am a citizen of the United States of America residing at 34 Brenton Road, Weston, Massachusetts 02193.

2. I graduated from University of Maryland in 1969 with a Bachelor of Science degree. In 1973 I received an MD degree from Tufts University School of Medicine. A copy of my curriculum vitae was attached to my Declaration filed December 12, 1995.

3. I am currently a professor of Medicine and Cardiology at Tufts University School of Medicine, Boston, Massachusetts, as well as Chief of Cardiovascular Research at St. Elizabeth's Medical Center, Boston, Massachusetts. I have held these positions since 1988.

4. I am also the Director of the Human Gene Therapy Laboratory at St. Elizabeth's Medical Center.

5. Attached to this Declaration as Attachment A is a reprint from the August 10, 1996 issue of the Lancet that was discussed with Examiner Low on November 16, 1996 at an interview. This document accurately reflects work that was done under my supervision and control. The publication documents anglographic and histologic evidence of anglogenesis in the ischemic limb of a 70-year-old woman with gangrene of the right great toe after using the present invention. We found by digital subtraction angiography that 4 weeks after gene therapy began the patient showed an increase in collateral vessels in the ischemic limb at the knee, mid-tibial, and ankle levels (figure 2). This was still shown at a 12-week contrast angiogram. The gene therapy augmented collateral network was associated with an increase in resting and maximum blood flow. After we

delivered a gene encoding an angiogenesis promoting molecule having a secretory signal (i.e., phVEGF) to the patient, flow measured at rest was 82% greater than that measured immediately before gene transfer, while maximum flow was 72% greater.

Another indication that our methodology had promoted angiogenesis was inspection of the integument of the distal portion of the ischemic limb. Three spider angiomas developed over the medial ankle (1) and dorsal forefoot (2) about a week after gene transfer (figure 3). Excisional biopsy and light microscopy (figure 3) of one of these lesions disclosed positive staining for CD31. Such positive staining identifies endothelial cells comprising vessels within this lesion. The proliferative nature of these endothelial cells was shown by immunostaining of adjacent sections for proliferating-cell nuclear antigen. The two lesions not removed by surgical biopsy spontaneously regressed by 8 weeks after gene transfer.

6. This work was also the subject of an article in the Science section of the August 27, 1996 *New York Times*. A copy of this article is attached to the Declaration as Attachment B. As set forth in the article, other doctors involved in cardiology research commented on our work. For example, Dr. Steven E. Nissen, vice-chairman of the division of cardiology at the Cleveland Clinic Foundation stated that "[t]his is really monumentally important. This is an entirely new approach [with] implications that are very broad."

7. Also discussed at the interview were the results seen with other patients in the clinical study. Attached to this Declaration as Attachment C are photographs documenting continued success of the present invention in inducing the formation of new blood vessels in a desired target tissue, e.g., an ischemic limb.

Fig. 1 shows magnetic resonance angiography (MRA, performed without contrast media) pre- and post-gene therapy in patient (Pt.) 5. In comparison to the longitudinal reconstruction (top) and tomographic view of the infrapopliteal vessels recorded pre-gene therapy, the study recorded post-gene therapy shows enhanced flow via a tortuous collateral (arrow) to the peroneal (Per) artery distally; flow into the anterior (AT) artery as improved as well.

Fig. 2. shows MRA pre- and post-gene therapy in PT. 6: post gene therapy there is improved flow in the deep femoral (DF) artery, as well as the posterior tibial (PT) and peroneal (Per) arteries.

Fig. 3. shows MRA pre- and post-gene therapy in Pt. 7: post-gene therapy, markedly improved flow is seen in the posterior tibial (PT) and peroneal (Per) arteries, and, to a lesser degree, in the anterior tibial (AT) artery as well.

Fig. 4. is a one-year MRI follow-up of Pt. 5 showing persistent improvement in peroneal artery blood flow.

8. We have found that the presence of secretory sequences on the angiogenesis-promoting compounds used is an important factor. For example, native aFGF (acidic Fibroblast Growth Factor) promotes angiogenesis. However, its effectiveness when used in gene therapy *in vivo* in promoting such angiogenesis has been disappointing. Native aFGF does not contain a secretory signal. We created a version that had a secretory signal and used that for *in vivo* gene transfer. Attached to the Declaration as Attachment D is a rough draft of a manuscript setting forth experimental results demonstrating that, following gene transfer of such DNA encoding an angiogenic protein, aFGF, engineered to have a secretory signed sequence, new blood vessel formation was induced *in vivo*. This document accurately reflects work under my supervision and control.

9. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 11/18/96

Jeffrey M. Isner

wp#51608

THE LANCET

Clinical evidence of anglogenesis after arterial gene transfer of phVEGF$_{165}$ in patient with Ischaemic limb

Jeffrey M Isner, Ann Pieczek, Robert Schainfeld, Richard Blair, Laura Haley, Takayuki Asahara, Kenneth Rosenfield, Syed Razvi, Kenneth Walsh, James F Symes

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Attachment A

THE LANCET

Clinical evidence of angiogenesis after arterial gene transfer of phVEGF$_{165}$ in patient with ischaemic limb

Jeffrey M Isner, Ann Pieczek, Robert Schainfeld, Richard Blair, Laura Haley, Takayuki Asahara, Kenneth Rosenfield, Syed Razvi, Kenneth Walsh, James F Symes

Summary

Background Preclinical findings suggest that intra-arterial gene transfer of a plasmid which encodes for vascular endothelial growth factor (VEGF) can improve blood supply to the ischaemic limb. We have used the method in a patient.

Methods Our patient was the eighth in a dose-ranging series. She was aged 71 with an ischaemic right leg. We administered 2000 μg human plasmid phVEGF$_{165}$ that was applied to the hydrogel polymer coating of an angioplasty balloon. By inflating the balloon, plasmid DNA was transferred to the distal popliteal artery.

Findings Digital subtraction angiography 4 weeks after gene therapy showed an increase in collateral vessels at the knee, mid-tibial, and ankle levels, which persisted at a 12-week view. Intra-arterial doppler-flow studies showed increased resting and maximum flows (by 82% and 72%, respectively). Three spider angiomas developed on the right foot/ankle about a week after gene transfer; one lesion was excised and revealed proliferative endothelium, the other two regressed. The patient developed oedema in her right leg, which was treated successfully.

Interpretation Administration of endothelial cell mitogens promotes angiogenesis in patients with limb ischaemia.

Lancet 1996; **348**: 370–74

Introduction

Among the growth factors that promote angiogenesis, vascular endothelial growth factor (VEGF),[1,2] also known as vascular permeability factor,[3] and vasculotropin,[4] is specifically mitogenic for endothelial cells. The first exon of the VEGF gene includes a secretory signal sequence that permits the protein to be secreted naturally from intact cells.[1] We have shown[5,6] that arterial gene transfer of naked DNA encoding for secreted protein yielded physiological levels of protein despite low transfection efficiency. Site-specific gene transfer of plasmid DNA encoding the 165-aminoacid isoform of human VEGF (phVEGF$_{165}$) applied to the hydrogel polymer coating of an angioplasty balloon,[7] and delivered percutaneously to the iliac artery of rabbits in which the femoral artery had been excised to cause unilateral hindlimb ischaemia led to development of collateral vessels and increased capillary density, improved calf blood-pressure ratio (ischaemic/normal limb) and increased resting and maximum vasodilator-induced blood flow.[8,9] We now use this strategy in the ischaemic limb of a patient.

Departments of Medicine, Biomedical Research, Radiology, and Surgery, St Elizabeth's Medical Center, Tufts University School of Medicine, Boston, Massachusetts, USA (Jeffrey M Isner MD, Ann Pieczek BA, Robert Schainfeld DO, Richard Blair MD, Laura Haley BS, Takayuki Asahara MD, Kenneth Rosenfield MD, Syed Razvi MD, Kenneth Walsh PhD, James F Symes MD)

Correspondence to: Dr Jeffrey M Isner, St Elizabeth's Medical Center, Boston, MA 02135, USA

Patient and methods

Patient

A 70-year-old non-diabetic woman was referred for gangrene of the right great toe. About a year earlier, the patient had cramping right-foot pain; several corns were removed, she was given intramuscular cortisone, prescribed ibuprofen, and fitted with shoe inserts. Symptoms worsened and the patient received oxycodone, hydrocodone, and a fentanyl patch. The great toe lesion progressed to gangrene, and the second and third toes became compromised. She had no palpable pedal pulses of the right limb. Ankle-brachial index of the ischaemic limb was 0·26. Arteriography revealed a 40% stenosis of the proximal popliteal artery, and occlusion of the peroneal, anterior tibial, and posterior tibial arteries midway to the foot. Surgical exploration of the distal right limb failed to identify a suitable site for a bypass.

The patient was suitable for arterial gene therapy according to a protocol[10] approved by the Human Institutional Review Board and Institutional Biosafety Committee of our centre, as well as the Recombinant DNA Advisory Committee of the National Institutes of Health and the US Food and Drug Administration.

Plasmid DNA

phVEGF$_{165}$ consists of a eucaryotic PUC 118 expression vector into which cDNA encoding the 165-aminoacid isoform of VEGF has been inserted.[11] A 763 basepair cytomegalovirus promoter/enhancer is used to drive VEGF expression. The PUC 118 vector includes an SV40 polyadenylation sequence, an *Escherichia coli* origin of replication, and the β-lactamase gene for ampicillin resistance. The plasmid was prepared in the Human Gene Therapy Laboratory at our centre from cultures of phVEGF$_{165}$-transformed *E coli*, purified with a Qiagen-tip 2500 column, precipitated with isopropanol, washed with 70% ethanol, and dried on a Speed Vac. The purified plasmid was reconstituted in sterile saline, stored in vials, and pooled for quality control analyses (absorbance at wavelengths of 260 and 280 nm to document ratio between 1·75 and 1·85; limulus amoebocyte lysate gel-clot assay [BioWhittaker] to establish bacterial endotoxin levels below 5 endotoxin units per kg bodyweight; microbial cultures; southern blot for level of contaminating genomic *E coli* DNA; and ethidium bromide staining after agarose-gel electrophoresis to confirm that over 90% of the nucleic acid was in the closed, circular supercoiled form). To confirm the identity of the prepared plasmid, the VEGF-coding region from each pooled batch was resequenced (Applied Biosystem 373A).

Percutaneous arterial gene transfer

Arterial gene transfer was done with a hydrogel-coated balloon-angioplasty-catheter (Boston Scientific).[7] A sterile pipette was used to apply 2000 μg plasmid DNA at 10·3 μg/μL in 194·2 μL

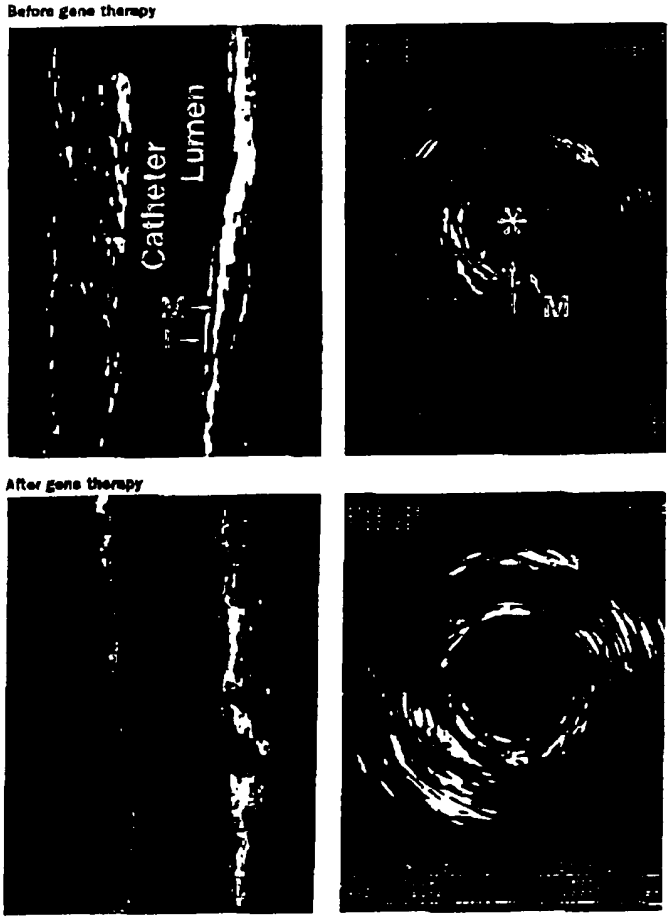

Figure 1: **Three-dimensional reconstructions of entire length of gene-transfer site**
Top=before, and bottom=after arterial gene transfer. Uniform luminal calibre is preserved, with pristine, three-layer appearance of arterial wall with no intimal thickening. I=intima, M=media, *=ultrasound catheter.

sterile saline to the external hydrogel coat of the inflated angioplasty balloon. The balloon was deflated, retracted into a protective sheath, reinflated to 2280 mm Hg, and advanced along with the sheath over a 45·7 mm guidewire under fluoroscopic guidance to the site of gene transfer. The balloon was then deflated, the sheath retracted, and the balloon reinflated at nominal pressures for 4–5 min. The balloon was deflated, all catheters and wires removed, and a final angiogram recorded to ensure satisfactory patency of the site.

Results

Intravascular ultrasound immediately before gene transfer showed that the intended site, the distal popliteal artery, was free of atherosclerotic plaque that might compromise transfection efficiency.[12] The arterial wall had a clearly recognisible three-layer appearance with no intimal thickening.[13] Repeat ultrasound at 4 weeks and 12 weeks after gene transfer disclosed no neointimal thickening resulting from inflation of the hydrogel-coated angioplasty-balloon-catheter (figure 1).

Digital subtraction angiography 4 weeks after gene therapy showed an increase in collateral vessels in the ischaemic limb at the knee, mid-tibial, and ankle levels (figure 2), which persisted at a 12-week contrast angiogram. The augmented collateral network was associated with an increase in resting and maximum flow.[14] After gene transfer, flow measured at rest was 82% greater than that measured immediately before gene transfer, while maximum flow was 72% greater. These findings are similar to those previously documented after treatment with rhVEGF protein in a rabbit model of hindlimb ischaemia.[14] Moreover, magnetic resonance angiography at 4 and 12 weeks after gene transfer gave qualitative confirmation of improved flow distal to the pre-existing total occlusions of the peroneal and tibial arteries.

Angiogenesis was also apparent on inspection of the integument of the distal portion of the ischaemic limb. Three spider angiomas developed over the medial ankle

Figure 2: **Selective digital subtraction angiography immediately before (left) and 1 month after (right) gene therapy**
New collateral vessels are visible.

(1) and dorsal forefoot (2) about a week after gene transfer (figure 3). Excisional biopsy and light microscopy (figure 3) of one of these lesions disclosed positive staining for CD31, which identifies endothelial cells comprising vessels within this lesion. The proliferative nature of these endothelial cells was shown by immunostaining of adjacent sections for proliferating-cell nuclear antigen. The two lesions not removed by surgical biopsy spontaneously regressed by 8 weeks after gene transfer.

Transient peripheral oedema was also observed in the ischaemic limb about 7 days after the procedure. Ankle circumference increased from 8·5 to 9·5 cm. The oedema was treated with bumetanide 1–3 mg per day as needed and resolved by 4 weeks.

Despite augmented collateral flow, limb gangrene could

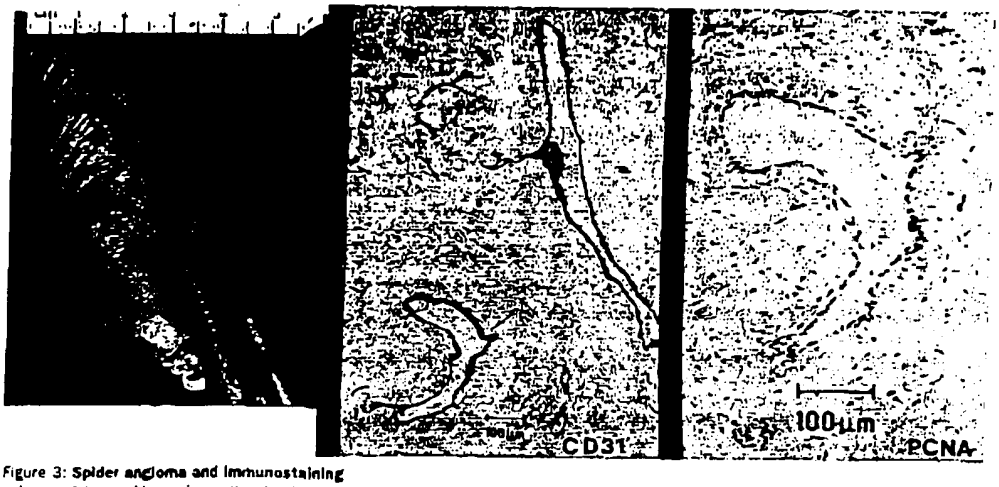

Figure 3: **Spider angioma and immunostaining**
Left-one of three spider angiomas that developed about 1 week after gene therapy in distal portion of ischaemic limb. Middle and right-tissue sections stained with antibody to endothelial antigen CD31 show vascularity of lesion, while stain of adjacent section for proliferating-cell nuclear antigen (PCNA) shows extent of proliferative activity among endothelial cells in lesion.

not be reversed, and the patient required below-knee amputation 5 months after gene therapy.

Discussion

Beginning with Nabel et al,[1] several laboratories showed the feasibility of arterial gene transfer in animal models. In December, 1994, we began a dose-escalating clinical trial.[11] Doses of 100 and 500 μg of phVEGF$_{165}$ administered to one patient each were inactive. 1000 μg improved lower extremity blood-flow in three of five patients, revealed by intravascular doppler analysis and magnetic resonance imaging. Contrast angiography, however, disclosed no new collateral vessels. DNA-labelling studies in pig and dog models of myocardial ischaemia and in the rabbit ischaemia hindlimb model[16-18] established that improvement in collateral-dependent flow typically results from proliferation of new vessels of under 180 μm in diameter. So we inferred that flow from the profunda to the infrapopliteal vessels was improved via an augmented network of collaterals that were too small to be seen on conventional angiography. Direct angiographic evidence of an augmented collateral network was observed for the first time in the current patient, the eighth in our series and the first to receive 2000 μg plasmid DNA.

The time course of angiogenesis documented by digital subtraction angiography and skin biopsy in the current patient is consistent with observations on the course of gene expression in the rabbit ischaemic hindlimb following hydrogel-balloon transfection of the internal iliac artery; reverse-transcription PCR confirmed reproducible gene expression at the mRNA level for up to 21 days after gene transfer.[16] The patient's improved blood flow in the transfected limb, as shown by magnetic resonance imaging and intra-arterial doppler analysis, suggest that the strategy of therapeutic angiogenesis is clinically feasible. The principal challenge now is to optimise this strategy to achieve limb salvage.

Clinical studies of VEGF recombinant protein are currently not possible because the protein is not available for human application. Should protein therapy become an option, however, it will be intriguing to see whether slow-release gene therapy, as we did site-specifically and targeted to local pathology, proves superior for bioactivity and/or safety to bolus or continuous protein administration.

The site of balloon inflation was selected specifically because intravascular ultrasound disclosed no evidence of atherosclerotic plaque that might compromise the efficiency of gene transfer.[17] The improvement in blood flow after gene transfer cannot therefore be attributed to reduction in luminal narrowing by balloon angioplasty since the baseline appearance was normal. Neither of the ultrasonograms after gene therapy showed intimal thickening. phVEGF$_{165}$ gene transfer accelerates re-endothelialisation, and thereby obviates neointimal thickening of the transfected segment.[11]

The spider angiomas that developed in the foot and ankle distal to the site of gene transfer are strong, albeit indirect, evidence of gene expression in our patient. Immunohistochemical staining of the lesion that was resected showed extensive endothelial proliferation. These lesions were first observed a week after gene transfer and regressed by 7 weeks later. The lesions were limited to the distal portion of the ischaemic limb, perhaps by localised upregulation of endothelial VEGF receptors.[20] The time course and distribution of these lesions suggested that these vascular malformations developed as a consequence of phVEGF$_{165}$ expression. The lesions were reminiscent of supernumerary vessels described after injection of VEGF recombinant protein into quail embryos.[21,22] These lesions, although benign and in our case self-limited, may represent unwanted angiogenesis, and warrant careful monitoring in future patients who receive angiogenic therapy.

Our patient also developed unilateral peripheral oedema after gene transfer. VEGF increases vascular permeability,[23] hence its alternative name, vascular permeability factor.[3] The fact that oedema developed for the first time after gene therapy and resolved by week 5 post-gene transfer (1 week beyond the 21–30 days[11] during which the transgene is expressed) suggests that this finding was also a consequence of phVEGF$_{165}$ gene transfer. The association between neovascularity and oedema in our patient is consistent with previously reported evidence[23,24] that linked VEGF as a vascular growth factor and permeability factor.

In our protocol, the requirement for a higher transfection efficiency may be obviated by the fact that VEGF protein includes a leader sequence which permits active secretion from intact cells. Thus, even if VEGF gene expression is limited to a few cells, the paracrine effects of the secreted gene product may be sufficient to achieve a meaningful biological effect. Whether higher doses of naked plasmid DNA will suffice or whether despite the secreted feature of the gene product, the magnitude of gene expression required will demand the use of adjunctive, including viral, vectors remains to be addressed. If naked plasmid DNA alone is to be used, then the optimum dose of plasmid DNA remains to be established. Other critical issues include the best frequency of administration. If the gene product is limited to less than 30 days, as suggested by preclinical studies,[16,19] the time interval required for full maturation of a lengthy collateral network might benefit from repeated administration (eg, 3 weeks) after the first dose. The extent to which a favourable response is affected by the proximity of the site of gene transfer to the ischaemic focus also remains uncertain.

We thank Susan Rossow for preparation of the plasmid DNA, Jason Lowey for re-sequencing of the plasmid DNA, Joachim Schorr (Qiagen) for quality control testing of the plasmid, Jeff Griffiths for supervising the microbial testing of the plasmid, Kathleen Hogan, Dave Cave, and Jay Duker for evaluating the patient's suitability for arterial gene therapy, and Susan Kelly, patient's advocate.

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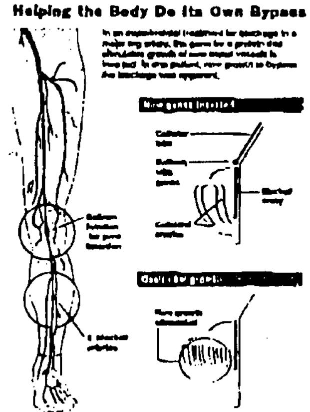
Gene Therapy Shows First Signs Of Bypassing Arterial Blockage

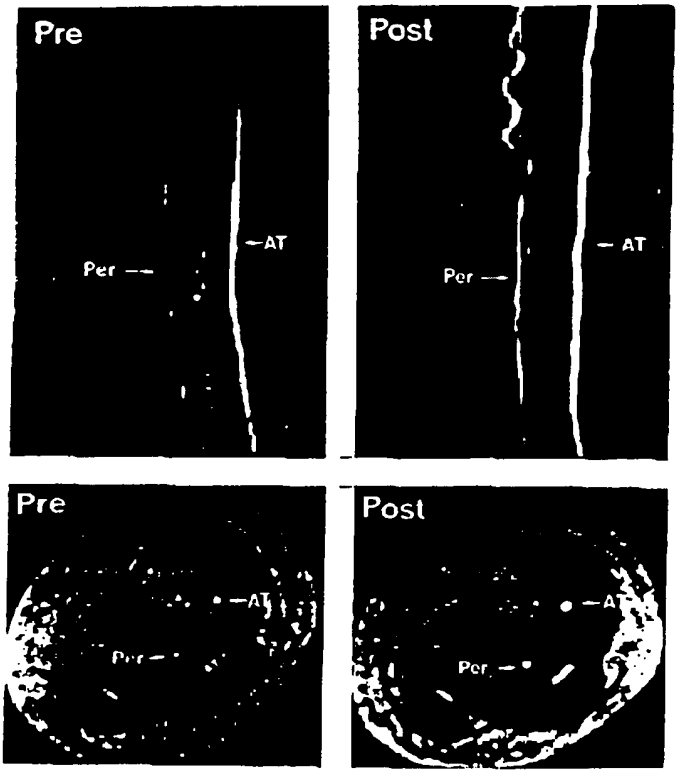
Helping the Body Do Its Own Bypass



New genes helped a patient's blood supply but did not prevent an amputation.

Progress is made in a field that has been full of promise but short of results.

Figure 1



Attachment C

Figure 2

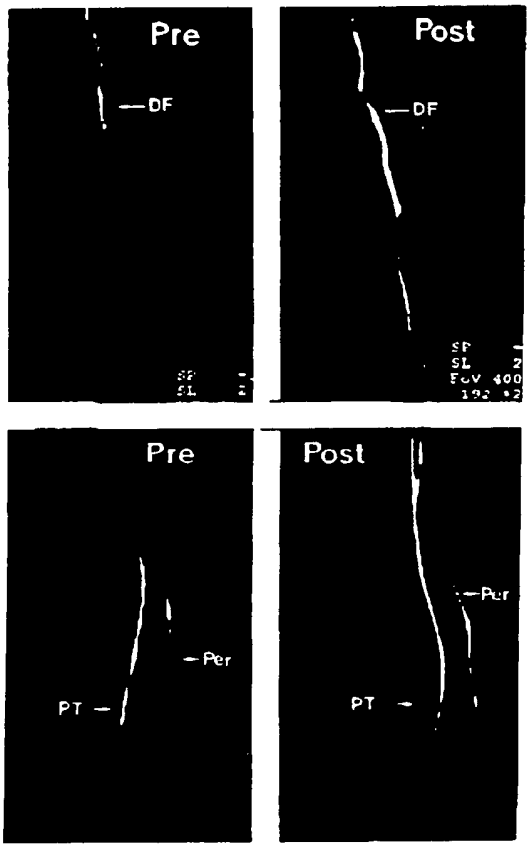


Figure 3

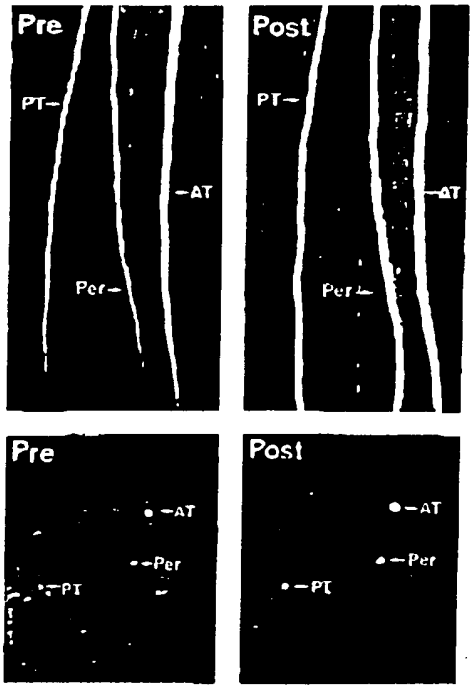
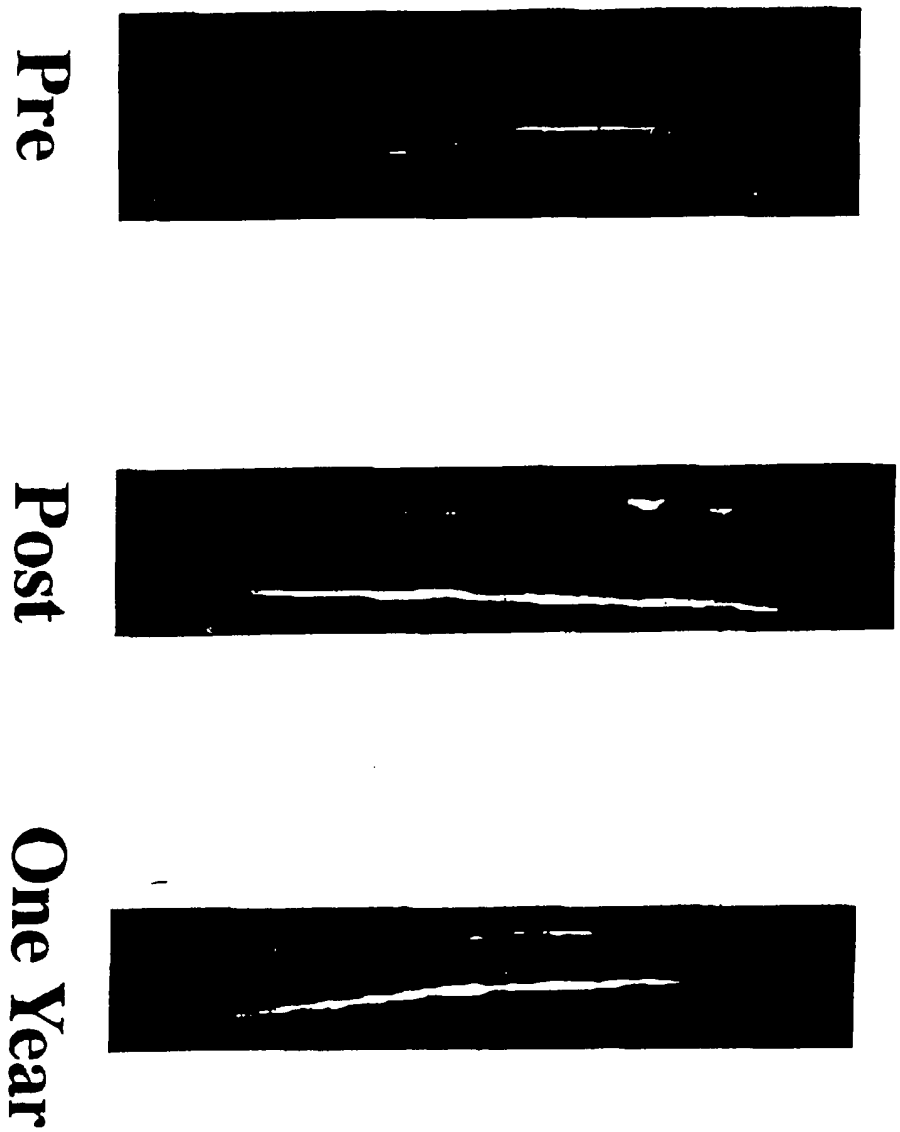


Figure 4



Arterial Gene Transfer of Acidic Fibroblast Growth Factor for Therapeutic Angiogenesis in vivo: Critical Role of Secretion Signal in Use of Naked DNA

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Abstract

Background Previous studies have demonstrated that heparin-binding growth factors including acidic (aFGF) and basic (bFGF) fibroblast growth factor could promote angiogenesis. However, in vivo, the role of aFGF, that is lack of secreted signal originally, in vascular response has been difficult to determine. Therefore, we investigated the hypothesis that the gene transfer with naked aFGF DNA encoding secret signal sequence could achieve therapeutic angiogenesis in vivo.

Methods and results At ten days following surgical induction of unilateral hindlimb ischemia, New Zealand white rabbits were randomized to be performed intra-arterial gene transfer via a hydrogel balloon catheter in the internal iliac artery of the ischemic limb; 500 μg of pMJ35 (secreted type aFGF DNA)(n=10), 500 μg of p267 (non-secreted type aFGF DNA)(n=10), or 500 μg of pGSVLacZ (n=10). At day 30 post-gene transfer, pMJ35 transfectans had more angiographically visible collateral (angiographic score=0.76±0.02) vs p267 (0.55±0.02, $p<0.01$) vs LacZ (0.47±0.02, $p<0.001$). Calf blood pressure ratio for pMJ35 was 0.88±0.02 vs 0.68±0.04 for p267 ($p<0.01$) and 0.57 ±0.04 in LacZ ($p<0.001$). Capillary density (per square mm) was also superior in pMJ35 group (274±10) vs p267 (204±9, $p<0.01$) and LacZ (177±6, $p<0.001$). Flow to ischemic limb measured by intra-aortic Doppler wire at day 30 post-transfer showed that maximum flow (ml/min) after administration of nitroprusside was highest in pMJ35 group (49.6±7.6, $p<0.05$) vs LacZ (25.0±5.7). Perfusion of ischemic muscles, including adductor, semimembranosus, and gatrocunemius muscle, assessed by colored microsphere were also highest in pMJ35 group. RT-PCR disclosed human aFGF mRNA in transfected arteries of pMJ35 but not LacZ up to 21 days post-gene transfer.

Conclusion Optimal outcome for therapeutic angiogenesis following arterial gene transfer of naked aFGF DNA depends on the presence of secret signal sequence, as paracrine effects of secreted protein.

Introduction

Ischemic diseases represent the major cause of morbidity and mortality, and revascularization such as angioplasty or bypass surgery is undertaken commonly. However, revascularization of small or diffuse arteries is usually unsuccessful and restenosis or reocclusion after revascularization of relatively large arteries are still problem. Under these conditions, therapeutic strategies designed to argument native collateral vessel blood flow represent desirable means.

Acidic fibroblast growth factor (aFGF), which is mitogenic for endothelial cells, induces the components of vascular growth in vitro and stimulates the angiogenesis in vivo [1-3]. In addition, aFGF may play a role in the differentiation of the mesoderm during development [4] and shares sequence similarity with several oncogenes [5]. It is also reported that aFGF may both promote repair of damaged endothelium and inhibit the accompanying intimal thickening [6]. However, unlike most polypeptide growth factors, the structure of the aFGF translation product dose not possess an apparent classical secretory signal sequence [7-8] and aFGF open reading frame is flanked by termination codons [7]. A secreted form of the aFGF gene was derived by ligation of the signal sequence from the human fibroblast interferon to the first residue (methionine) of the encoded aFGF [9]. Though the site-specific transfection efficiency using catheter is relatively low, the gene encoding secreted protein may achieve therapeutic effect.

Accordingly, in the current study, we investigated the hypothesis that site-specific transfection of the plasmid encoding aFGF with secreted sequence could stimulate angiogenesis and modulate the hemodynamic deficit in chronic ischemic limb model, compared with the plasmid encoding aFGF without secreted sequence and control plasmid. The outcome of these in vivo experiments, performed in a rabbit model, resulted in therapeutic angiogenesis; evidence for this therapeutic endpoint included augmented

development of collateral vessels, marked reduction of hemodynamic deficit and increased capillary density.

Methods

Animal model

We used a rabbit ischemic hindlimb model that has been described previously [10], to investigate the angiogenic response. All protocols were approved by St. Elizabeth's Institutional Animal Care and Use Committee. Thirty male New Zealand White rabbis weighting 4-4.5 kg (Pine Acre Rabbitry, Norton, MA) were anesthetized with a mixture of ketamine (50 mg/kg) and acepromazine (0.8 mg/kg) following premedication with xylazine (2.5 mg/kg). A longitudinal incision was then performed, extending inferiorly from the inguinal ligament to a point just proximal to the patella. The limb in which the incision was performed - right versus left - was determined at random at the time of surgery by the operator. Through this incision, with surgical loops, the femoral artery was dissected free along its entire length; all branches of the femoral artery, including the inferior epigastric, deep femoral, lateral circumflex and superficial epigastric arteries, were also dissected free. After further dissecting the popliteal and saphenous arteries distally, the external iliac artery as well as all above arteries were ligated with 4.0 silk (Ethicon, Sommerville, NJ). Finally, the femoral artery was completely excised from its from its proximal origin as a branch of the external iliac artery, to the point distally where it bifurcates into the saphenous and popliteal arteries. The excision of the femoral artery results in retrograde propagation of thrombus to the origin of the external iliac artery. Consequently, blood flow to the ischemic limb is dependent upon collateral vessels which may originate from the internal iliac artery.

Recombinant Plasmid

The recombinant expression vector p267 (paFGF), containing the human aFGF cDNA, encodes the 16-kDa aFGF [11]. The cDNA sequence was cloned under the control of the simian virus 40 early promotor (SV40 ep) and the cytomegalovirus enhancer (CMV enh.), with downstream SV40 regulatory sequences. A similar construct, pMJ35 (pSP-aFGF), containing 5' to the aFGF cDNA a heterologous SP sequence coding for 21 amino acids was constructed. This construct joints the SP of human fibroblast interferon [12] to the first residue (methionine) of the encoded aFGF (Fig.1) [9]. These recombinant plasmids, pMJ35 and p267, were a gift of Dr. Michael Jaye (Rhone-Poulenc Rorer central research, Collegeville, PA). The plasmid pGSVLacZ (courtesy of Dr. Claire Bonnerot) containing a nuclear targeted β-galactosidase sequence coupled to the simian virus 40 early promoter [13] was used for the control transfection experiment.

Percutaneous Arterial Gene transfer

An interval of 10 days between the time of surgery and the gene transfer was allowed for post-operative recovery of rabbits and development of endogenous collateral vessels. Beyond this timepoint, studies performed up to 90 days post-operatively [10] have demonstrated no significant collateral vessel augmentation. At 10 days post-operatively (day 0), after performing a baseline angiogram, the internal iliac artery of the ischemic limb was transfected with recombinant plasmid percutaneously using a 2.5 mm hydrogel-coated balloon catheter (Slider with Hydroplus, Boston Scientific, Watertown, MA) [14]. The angioplasty balloon was prepared (ex vivo) by first advancing the deflated balloon through a 5 Fr. Teflon sheath (Boston Scientific), applying of plasmid to the 20 μm-thick layer of hydrogel on the external surface of the inflated balloon, and then retracting the inflated balloon back into the protective sheath. The sheath and angioplasty catheter were then introduced to the lower abdominal aorta using a 0.014 inch guide wire (Hi-Torque Floppy II, Advanced Cardiovascular Systems, Temecula, CA) under fluoroscopic guidance. The

balloon catheter was then advanced into the internal iliac artery of the ischemic limb, inflated for 3 min at 6 atmospheres, deflated, and withdrawn. Heparin was not administered at the time of transfection or angiography.

A total 30 rabbits were transfected following recombinant plasmids; each 10 for 500 μg of pMJ35 (secreted type aFGF DNA), p267 (non-secreted type aFGF DNA), and pGSVLacZ, randomly.

Evaluation of Angiogenesis in the Ischemic Limb

Development of collateral vessels in the ischemic limb was serially evaluated by calf blood pressure measurement and internal iliac arteriography immediately prior to transfection (day 0), and then day 30 post-transfection. On each occasion, it was necessary to lightly anesthetize the animal with a mixture of ketamine (10 mg/kg) and acetapromazine (0.16 mg/kg) following premedication with xylazine (2.5 mg/kg). Following the final 30 day follow-up, the animal was sacrificed, and the tissue sections were prepared from the hindlimb muscles in order to perform analysis of capillary density and microsphere analysis. These analyses are discussed in detail below.

Calf Blood Pressure Ratio.

Calf blood pressure was measured in both hindlimbs using a Doppler Flowmeter (Model 1050, Parks Medical Electronics, Aloha, OR), immediately prior to transfection (day 0) as well as on day 30. On each occasion, the hindlimbs were shaved and cleaned; the pulse of posterior tibial artery was identified using standard techniques [15]. The calf blood pressure ratio was defined for each rabbits as the ratio of systolic pressure of the ischemic limb to systolic pressure of the ischemic limb to systolic pressure of the normal limb.

Selective Internal Iliac Arteriography.

Collateral artery development in this ischemic hindlimb model originates from the internal iliac artery. Accordingly, selective internal iliac arteriography was performed on day 0 (immediately prior to transfection) and on day 30 post-transfection as previously described [15]. A 3 Fr. end-hole infusion catheter (Tracker-18, Target Therapeutics, San Jose, CA) was introduced into the common carotid artery through a small cutdown, and advanced to the internal iliac artery of the ischemic limb using a 0.014 inch guide wire (Hi-Torque Floppy II) under fluoroscopic guidance. The tip of the catheter was positioned in the internal iliac artery at the level of the interspace between the seventh lumber and the first sacral vertebrae. Following intra-arterial injection of nitroglycerin (0.25 mg, SoloPark Laboratories, Franklin Park, IL), a total of 5 ml of contrast media (Isovue-370, Squibb Diagnostics, New Brunswick, NJ) was then injected using an automated angiographic injector (Medrad, Pittsburgh, PA) programmed to reproducibly deliver a flow rate of 1 ml per sec. Serial images of the ischemic hindlimb were recorded on 105-mm spot film at a rate of 1 film per sec for at least 10 sec. Following completion of arteriography, the catheter was removed and the wound was closed. All of the above-described procedures were completed without the use of heparin.

Morphometric angiographic analysis of collateral vessel development was performed on the 4-second angiogram. To assess the number of collateral vessels, we used a grid overlay comprised of 2.5 mm-diameter circles arranged in rows spaced 5 mm apart. This acetate overlay was placed over the angiogram recorded at the level of the medial thigh. The number of contrast-opacified arteries crossing over circles as well as the total number of circles encompassing the medial thigh area were counted by a single observer blinded to the treatment regimen. An angiographic score was calculated for each film as the ratio of grid intersections crossed by opacified arteries divided by the total number of grid intersections in the medial thigh.

Measurement of blood flow in ischemic hindlimb using Doppler

Blood flow was assessed on day 30 in the ischemic hindlimb with use of a Doppler guide wire, immediately before the selective internal iliac areteriography. As previously described [16-19], an 0.018 inch Doppler guide wire (Cardiometrics, Inc., Mountain View, CA) was advanced through the 3F infusion catheter (Tracker-18) to the proximal segment of the internal iliac artery supplying the ischemic limb. The Doppler wire recorded a real-time spectral analysis of the Doppler signal, from which the average velocity (APV) (the temporal average of the instantaneous peak velocity waveform) was calculated and displayed on-line. A second 3F perfusion catheter was introduced into the common carotid artery through the same cutdown and advanced under fluoroscopic guidance to the origin of the common iliac artery of the ischemic limb with a 0.014 inch guide wire (Hi-Torque Floppy II). This catheter was used for infusion of sodium nitroprusside (Sigma, St. Louis, MO), for direct measurement of intra-arterial blood pressure via connection to a pressure transducer (model 78534C, Hewlett Packard, Andover, MA), and for selective angiography of the ischemic limb. Blood pressure was monitored before and immediately after sodium nitroprusside administration. Angiography was performed before and after the drug administration with 1 ml of contrast media (Isovue-370). Serial images of the ischemic limb were recorded on 105-mm spot film at a rate of two films per second for 5 seconds. Sodium nitroprusside was administered intra-arterially via a constant infusion pump (1ml/min) at the doses of 1.5 μg/kg/min for 2 minutes, as previously reported.[20-21]

The vascular diameter was measured at the site of the Doppler sample volume (5mm distal to the wire tip [16]). Cross-sectional area was calculated assuming a circular lumen. Doppler-derived flow was calculated as $Q_D=(\pi d^2/4)(0.5\times APV)$, where Q_D is Doppler-derived time average flow (ml/min), d is vessel diameter, and APV is time average of the spectral peak velocity.[16] The mean velocity was estimated as 0.5×APV by assuming a time-averaged parabolic velocity profile across the vessel. Also vascular resistance was calculated as $R_E=P_E/Q_D$, where R_E is vascular resistance and P_E is intra-arterial blood pressure.

Capillary density and capillary/muscle fiber ratio.

The angiogenic effect of aFGF at the microvascular level was examined by measuring the number of capillaries in light microscopic sections taken from the ischemic hindlimbs. Tissue specimens were obtained as transverse sections from the adductor muscle and the semimembranosus muscle of both limbs of each animal at the time of sacrifice (day 30 post-transfectiont). These two muscles were chosen for light microscopic analysis because (1) they are 2 major muscles of the medial thigh, and (2) each was originally perfused by the deep femoral artery, ligated at the time that the common/ superficial femoral artery was excised. Muscle samples were embedded in O.C.T. compound (Miles, Elkhart, IN) and snap frozen in liquid nitrogen. Multiple frozen sections (5 μm in thickness) were then cut from each specimen on a cryostat (Miles), so that the muscle fibers were oriented in a transverse fashion, and two sections were then placed on glass slides. Tissue sections were stained for alkaline phosphatase using an indoxyl-tetrazolium method to detect capillary endothelial cells as previously described,[22] and were then counterstained with eosin. Capillaries were counted under 20x objective to determine the capillary density (mean number of capillaries per mm^2). A total of 20 different fields from the two muscles were randomly selected, and the number of capillaries counted. To ensure that analysis of capillary density was not overestimated due to muscle atrophy, or underestimated due to interstitial edema, capillaries identified at necropsy were also evaluated as a function of muscle fibers in histologic section. The counting scheme used to compute the capillary/muscle fiber ratio was otherwise identical to that used to compute capillary density.

Measurement of regional blood flow by colored microsphere

To evaluate the therapeutic effect on regional blood flow of treated muscle, colored microspheres (Dye-Track, Triton Technology, Inc., San Diego, CA) [20,23] were injected

into left ventricle at day 30 immediately before measurement of Doppler guide wire and angiogram. All procedures were performed according to the manufacturer's instructions. Reference arterial blood samples were collected through the aortic catheter starting 10 s before injection of the microspheres and continuing 120 s at the rate of 2 ml/min. Each more than two tissue specimens were obtained from the adductor muscle, the semimembranosus muscle, and the gastrocnemius muscle at the time of sacrifice. Samples were placed into teflon-sealed 16 ml screw-cap tubes and 7 ml of a 4 molar KOH solution containing 2% Tween 80 were added to each sample for digestion of the tissue. The digested sample solution was performed vacuum filtration using a polyester filter (Triton Technology, Inc., San Diego, CA). The tightly folded filter, trapping the spheres, was placed in a 1.5 ml Eppendorf tube and 150 µl of dimethyl-formamide were added. The container was capped and vortex mixed for 30 seconds, followed by 3 minutes of centrifugation at 2000 g. Then the solution was transferred to a spectrophotometer cuvette for photometric absorption analysis using a spectrophotometer (Model 8452A, Hewlett Packard, Andover, MA). Blood flow was calculated with the following equation:
Flow= flow rate of pump x (counts in sample/ g) x 100/ total counts in blood sample
=ml/ min/ 100 g tissue

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

We evaluated the time course expression of human aFGF mRNA using RT-PCR. Rabbit iliac arteries transfected with pMJ35 were harvested at days 3, 7, 14, 21, and 30 post-transfection (n=2 for each time point). Total cellular RNA was isolated from the sample using ULTRASPEC RNA (Biotecx Laboratories, Inc. Houston, TX) according to the manufacturer's instructions. Extracted RNA was treated with Dnase 1 (0.5 µl, 10 U/ µl, Rnase-free, Message Clean kit, GenHunter, Boston, MA) at 37 °C for 30 min to eliminate DNA contamination. The yield of extracted RNA was determined spectrophotometrically by ultraviolet absorbance at 260 nm. One µg of each RNA sample

was used to make cDNA in a reaction volume of 20 µl containing 0.5 mM of each deoxynucleotide triphosphate (Pharmacia, Piscataway, NJ), 10 mM dithioyhreitol, 20 units of Rnasin (Promega, Madison, WI), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 0.5 µg oligo primers (Promega), and 200 units of M-MLV reverse transcriptase (GIBCO BRL). Reactions were incubated at 42 °C for 1 hr, then 95 °C for 5 min to terminate the reaction.

Primers were designed according to conversed regions of the known human aFGF sequence [7]; aFGF-1 (5-AAT TAC AAG AAG CCC AAA CTC-3) and aFGF-2 (5-AGA CTG GCA GGG GGA GAA A-3) are homologous to the human aFGF sequence inserted to the pMJ35 and p267. In GeneAmp reaction tubes cDNA equivalent to 1 µg of total RNA was dissolved in 50 µl containing 1×PCR buffer, 2.5 mM $MgCl_2$, 25 pmoles aFGF-1 and aFGF-2 primer and 1.25 units Taq polymerase (Perkin Elmer, Branchburg, NJ). The PCR was performed on a 9600 PCR system (Perkin Elmer). Amplification was 30 step cycles: denaturing for 60 sec at 94 °C, annealing for 60 sec at 60 °C, and extension for 120 sec at 72 °C The final incubation was extended to 6 min. The sample was then rapidly cooled to 4°C and kept on ice until analysis by 1.5% agarose gel electrophoresis. DNA bands were visualized under UV illumination after staining with ethidium bromide.

To ensure that negative PCRs were not due to markedly different staring concentration of mRNA, PCR analysis for constitutively expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was performed on serial cDNA used in each PCR.

Evaluation of angiogenesis in dose-response with pMJ35

To evaluate the dose-response effect of pMJ35, we transfected 100, 200, 300, 400, 500, 700, 1000 µg of pMJ35 to the rabbit ischemic model (n=2 for each dose) and analyzed the calf blood pressure ratio, angiographic score, capillary density, and regional

blood flow using colored microsphere at 30 days after the transfection. All procedures were followed the same procedures mentioned above.

Statistical analysis

All results are expressed as mean± standard error (m±SE). Statistical comparisons were performed with the use of ANOVA. When a significant difference was detected, multiple-comparison analysis was performed using Scheffe's procedure. A value of $p<0.05$ was considered to denote statistical significance.

Results

Time Course of Human aFGF Gene Expression

To determine the time course of human aFGF gene expression, rabbit iliac arteries transfected with pMJ35 were harvested at days 3, 7, 14, 21, 30 post-transfection (n=2 for each timepoint), and analyzed for aFGF mRNA using RT-PCR. We used human fetus brain as positive control. Human aFGF mRNA expression was observed in the arteries harvested at day 3, 7 , 14, 21 post-transfection. In arteries harvested at day 30 post-transfection, however, aFGF gene expression was not observed. No aFGF gene expression was observed in the arteries transfected pGSVLacZ (Fig.2). In other organs, such as liver, heart, lung, spleen, testis, kidney, muscle, and non-transfected artery harvested at day 3 post-pMJ35 transfection, aFGF gene expression was not observed.

Angiographic Assessment

Fig.3 illustrates representative internal iliac angiogram at day 30, recorded from pMJ35, p267, and pGSVLacZ transfected animals. Serial assessment of number of angiographically visible collateral vessels (angiographic score as described above) showed a progressive increase throughout the follow-up period in all three groups. At baseline (day 0), there was no significant difference in angiographic score among the pMJ35, p267, and pGSVLacZ transfected groups (0.288± 0.01 vs 0.287± 0.01 vs 0.288 ±0.01, p=ns). By day 30, however, the angiographic score in pMJ35 transfected group (0.76± 0.02) was significantly higher than p267 group (0.55± 0.02, $p<0.01$) and pGSVLacZ group (0.47± 0.02, $p<0.001$) (Fig.4).

Calf Blood Pressure Ratio

Reduction of the hemodynamic deficit in the ischemic limb following pMJ35 transfection was confirmed by measurement of calf blood pressure ratio (ischemic/ normal limb). As shown in Fig.5, the calf blood pressure ratio was similar in all three groups prior to transfection (day 0) (0.39± 0.02 in pMJ35, vs 0.35± 0.02 in p267, vs 0.35± 0.02 in pGSVLacZ, p=ns). At day 30, the blood pressure ratio for pMJ35 transfected group (0.88± 0.02) was significantly higher than p267 group (0.68± 0.04, p<0.01) and pGSVLacZ group (0.57± 0.04, p<0.001).

Intravascular Doppler measurements in the internal iliac artery of the ischemic limb

At day 30, AVP and intra-aortic blood pressure were recorded at rest and after administration of sodium nitroprusside. At the same time, serial measurements of angiographic luminal diameter in the internal iliac artery at the site from which the Doppler measurements of flow were obtained. From these measurements, blood flow and vascular resistance were calculated. Although blood flow at rest was not significant different among three groups (24.8±3.6 ml/min in pMJ35, vs 16.6±2.2 in p267, vs 14.8±2.9 in pGSVLacZ), maximum flow provoked by intraaortic administration of nitroprusside was significantly higher in the pMJ35 group (49.6±7.6 ml/min) than in the pGSVLacZ group (25.0±5.7 ml/min)(p<0.05)(Fig.6a).

In contrast, vascular resistance was significantly lower in the pMJ35 group, compared with that in pGSVLacZ group both in resting state (3.2±0.4 vs 7.4±1.4 respectively, p<0.05) and after the administration of nitroprusside (1.5±02 vs 4.3±1.1 respectively, p<0.05)(Fig.6b).

Capillary Density and Capillary/ Muscle Fiber Ratio

A favorable effect of pMJ35 transfection on revascularization was also shown at the capillary level. The medial thigh muscles of the ischemic limbs were histologically

examined at day 30 after transfection, as described above. Mean value of capillary density in pMJ35 group (274± 10 /mm^2) was significantly higher than that in p267 group (204± 9 mm^2, p<0.01) and that in pGSVLacZ group (177± 6 mm^2, p<0.001) (Fig.7). Analysis of capillary/ muscle fiber ratio disclosed a value of 0.90± 0.02 in the pMJ35 group versus 0.66± 0.03 in the p267 group (p<0.001) and versus 0.54±0.02 in the pGSVLacZ group (P<0.001).

Measurement of Regional Blood Flow by Colored Microsphere

To further evaluate the effect of the transfection of pMJ35 on revascularization of the ischemic limb, Regional blood flow was assessed using colored microsphere method as mentioned above. In the ischemic adductor muscle, the regional blood flow of pMJ35 group (6.0± 1.7 ml/ min/ 100 g tissue) was significantly higher than that of pGSVLacZ group (1.9± 0.4, p<0.05) but there was no difference between that of p267 group (3.0± 0.4, p=ns). There were similar results in the ischemic semimembranosus muscle; 4.3± 0.7 in pMJ35 (p<0.05 vs pGSVLacZ),vs 2.7± 0.4 in p267, vs 1.8± 0.4 in pGSVLacZ, and in the ischemic gastrocnemius muscle; 4.7± 0.7 in pMJ35 (p<0.05 vs pGSVLacZ), vs 1.8± 0.3 in p267, vs 1.4± 0.4 in pGSVLacZ (Fig.8). There were no significant differences in normal limb blood flow measured by microsphere among all three groups.

Dose-Dependent Effect of pMJ35 for Angiogenesis

Fig.9 shows the evaluation of angiogenesis at day 30 after transfection of pMJ35 in each doses (0, 100, 200, 300, 400, 500, 700, 1000 μg). Angiographic score (Fig.9a), blood pressure ratio (Fig.9b), capillary density (Fig.9c), and measurement of regional blood flow by colored microsphere (Fig.9d) showed no significant effects using low dose of pMJ35 (100-300 μg), compared with control. In assessment of angiographic score, blood pressure ratio, and capillary density, the results of 500 μg pMJ35 were increased

significantly compared with control and the results were not increased further even using high dose (700-1000 μg) pMJ35. Only regional blood flow of gastrocnemius muscle assessing by colored microsphere (Fig.9d), showed increase in 1000 μg compared with 500 μg pMJ35 transfection (not significant).

Discussion

Despite major advances in both surgical and percutaneous revascularization techniques, therapeutic options for patients with obstructive vascular disease are limited.[24] Conventional drug therapy is not proven benefit for these patients. When vascular obstruction is lengthy and widespread or the artery is small, percutaneous revascularization may not be feasible. Surgical therapy is complicated by a variable morbidity and mortality, and is dependent on long-term graft patency. Under these conditions, therapeutic strategies to argument collateral blood flow constitutes a potential alternative treatment.

Recent studies have demonstrated the therapeutic potential of using various angiogenic growth factors to augment revascularization of the ischemic limb,[15,25-27] as well as myocardium.[28-30] Vascular endothelial growth factor,[15,27] Endothelial cell growth factor,[26] and basic FGF (bFGF)[25,27-28] have been demonstrated previously augmentation of angiographically visible collateral vessels, an increased number of capillaries, and consequent reduction of the hemodynamic deficit in the ischemic area. Previous studies have been suggested that aFGF is, as well as bFGF, directly involved in cardiac development and vasculogenesis.[4, 31-34] In addition, hypoxia is associated with increased expression of aFGF and hypoxia-mediated aFGF stimulates growth of endothelial cells.[35,36] Some papers have suggested that aFGF induces the components of vascular growth in vitro and stimulates the angiogenesis in vivo.[1-3,37]

However, unlike other angiogenic factors, the data of the angiogenic efficacy of aFGF to the ischemic area in vivo is limited. Bani demonstrated that aFGF protein delivery to the ischemic myocardium did not angiogenic response.[37] There are still some problems; the method of local delivery, the dose and the half-time of the protein, and the lack of secreted signal of original aFGF.[7-8] Now a secreted form of the human aFGF DNA has been derived by ligation of the signal sequence from the human fibroblast

interferon.[9] We therefore attempted to evaluate whether the site-specific transfection of the plasmid encoding the aFGF DNA with secreted sequence could stimulate angiogenesis and modulate the hemodynamic deficit in chronic ischemic limb model.

First of all, human aFGF mRNA expression in the rabbit artery after transfection of plasmid encoding aFGF DNA using hydrogel balloon technique, was observed at day 3, 7, 14, 21 post-transfection (Fig.2). The current findings that rabbit iliac arteries transfected with β-galactosidase gene was negative when examined for human aFGF mRNA and that other organs showed no aFGF mRNA expression at day 3 post-transfection of plasmid encoding aFGF DNA, supports that the primers did not cross-react with endogenous rabbit aFGF mRNA. Protein expression analysis techniques including Western method and immunohistochemical staining were found to be compromised by lack of available antibody to rabbit tissues.

In both the previous studies administration of recombinant protein or gene transfer, the angiographic , hemodynamic, and histologic extent of collateral development exceeded that observed in control animals at 30 days post-treatment. Previous studies in this animal model have established that the extent of natural (spontaneous) collateral development reaches a plateau at 30 days and remains unchanged up to 90 days follow-up.[10] In this current study, the improvement of angiographic score and of calf blood pressure ratio was statistically significant at day 30 post-transfection in secreted type aFGF gene (pMJ35) group, compared with non-secreted type aFGF gene (p267) and β-galactosidase gene (pGSVLacZ) group (Fig.4 and Fig.5). Anatomic evidence of neovascularity was investigated at two levels; at the capillary level and angiographically visible vessels level. Angiographic score reflects larger visible collateral arteries and serial measurements of the lower limb blood pressure reflects the physiologic equivalent of these anatomic findings, to which angiogenesis is therapeutic. The dramatic increase of blood pressure ratio in pMJ35 group is likely to be a consequence of better arterial reconstitution, compared with p267 group and pGSVLacZ group

Above conclusion is further supported by the maximum flow findings using Doppler guide wire (Fig.6). Blood flow at rest was similar in the three groups, in contrast, maximum flow provoked by intraarterial administration of sodium nitroprusside, a direct smooth muscle dilator, was significantly higher in the pMJ35 group (Fig.6a). These results were similar with previous studies,[38,39] which demonstrate that most models of chronic ischemia are characterized by a small deficit in resting flow, but maximum flow reserve, typically more severely and consistently impaired in these models, is more useful for investigating the physiologic impact of therapeutic angiogenesis. Thus in this current study, the significantly increased flow seen in the pMJ35 group after nitoprusside-induced vasodilation is a measure of the enhanced capacity of the treated limb blood flow as a result of enlarged capacity of the collateral bed to deliver blood downstream. This consideration is also supported by the results of vascular resistance (Fig.6b). The capillary level assessment of neovascularity showed the significantly increase of capillary density and capillary/muscle fiber ratio of the ischemic muscles in the pMJ35 group, compared with p267 and pGSLacZ groups (Fig.7a & 7b). These results reflect evidence that vascular adaptations in capillary level can be induced in response to the transfection of pMJ35 after ischemia.

In addition to the collateral flow measurement and capillary measurement, mentioned above, we directly evaluated the ishemic muscle perfusion using colored microsphere, in order to investigate whether pMJ35 transfection-induced vascular remodeling could improve blood flow to the distal tissue (muscle perfusion) most at risk for ischemia. As shown in Fig.8, in the group transfected pMJ35, the significant improvements of blood flow both in proximal hindlimb (Adductor and semimembranosus muscle) and in distal hindlimb (gastrocnemius muscle) were observed, compared with other two groups. Previous paper [40] reported that more than 2 weeks bFGF protein infusion into rat ischemic model improved blood flow in distal limb, but not in proximal limb, evaluating by microsphere technique. There could be several reasons getting

improvement of the blood flow in proximal limb by pMJ35; plasmid encoding secreted type aFGF DNA and non-secreted type bFGF protein, the method of delivery, modulation of the FGF receptor, and the deference of conduit vessel.

Direct arterial gene transfer has been previously demonstrated to be feasible,[14,41-45] and in certain cases capable of producing regional alterations in vascular wall biology.[3,46-47] Arterial gene transfer in this current study was performed without the use of transfection vehicle such as a liposome or viral vector. The use of "naked" DNA alone simplifies the transfection protocol and obviates concerns regarding the potential toxicity of liposome,[48] inactivation of liposomes by heparin and/or serum,[49] and reversion from replication-defective to infectious wild type in case of viral vectors. We considered the fact that the aFGF gene encodes a secretory signal sequence that might be exploited as part of a strategy designed to accomplish therapeutic angiogenesis by arterial gene transfer with the hydrogel-polymer-coated balloon catheter. We had previously used the plasmid pXGH5 encoding the gene for human growth hormone, a secreted protein, to transfer rabbit ear arteries in vivo [45] and rabbit aortic rings in vitro [50] and obtained for up to 3 weeks physiological level of human growth hormone, even though immunohistochemical examination of the transfected tissue disclosed evidence of successful transfection in <1% of cells in the transfected arterial segment.[14,50-51] Thus, gene products that are secreted may have profound biological effect, even when the number of transduced cells remains low.

Related to transfection efficiency, we preliminary evaluated the dose-response effect to the therapeutic angiogenesis using the plasmid encoded secreted type aFGF DNA (Fig.9). The results suggested that at least more than 400-500 μg plasmid would be needed to achieve the successful therapeutic angiogenesis, and that the effect of therapeutic angiogenesis would be plateau with high dose (700-1000 μg) plasmid. This could be related to the trasfection efficiency and to the modulation of the FGF receptor. According to these results, we used 500 μg plasmid of pMJ35, p267, and pGSVLacZ in this current study.

In conclusion, this study demonstrates that intraaortic transfection of plasmid encoding aFGF with secreted sequence produces a significant improvement in therapeutic angiogenesis in vivo. It has been suggested that the angiogenic efficacy of aFGF DNA transfection depends on the presence of secret signal sequence.

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Figure legends

Fig.1 Recombinant expression vectors.[9] The human aFGF cDNA sequence cloned in p267 has been reported.[11] The 422-base-pair cDNA sequence was cloned under the control of the simian virus 40 early promoter (SV40 ep) and the cytomegalovirus enhancer (CMV enh.), with downstream SV40 regulatory sequences. The pMJ35 recombinant was contains upstream of the first methionine codon of aFGF a heterologous sequence-coding for a signal peptide (SP), secreted signal sequence.

Fig.2a The expression of aFGF mRNA of the arteries transfected with pMJ35, using RT-PCR (Lane 3; 3 days, Lane 4; 7 days, Lane 5; 14 days, Lane 6; 21 days, Lane 7; 30 days post-transfection). Lane 1; human fetus brain as positive control. Lane 2; artery transfected pGSVLacZ.

Fig.2b RT-PCR analysis of aFGF mRNA 3 days after transfection with pMJ35. Lane 1; positive control. Lane 2; artery transfected with pGSVLacZ, Lane 3; liver, Lane 4; heart, Lane 5; lung, Lane 6; spleen, Lane 7; testis, Lane 8; kidney, Lane 9; muscle, Lane 10; non-transfected artery

Fig.3 Representative internal iliac angiogram at day 30 post-transfection. **a**; pMJ35, **b**; p267, **c**; pGSVLacZ

Fig.4 Effect of pMJ35, p267, and pGSVLacZ on angiographic score immediately before transfection (day 0) and at day 30 post-transfection (*$p<0.001$ vs pGSVLacZ group, #$p<0.01$ vs p267 group).

Fig.5 Effect of pMJ35, p267, and pGSVLacZ on calf blood pressure ratio (systolic pressure of the ischemic limb to systolic pressure of the normal limb) immediately before transfection (day 0) and at day 30 post-transfection (*p<0.001 vs pGSVLacZ group, #p<0.01 vs p267 group).

Fig.6a Blood flow (ml/min) at rest and at maximum flow (after administration of nitroprusside) of the ischemic limb assessed by intraaortic Doppler guide wire in pMJ35, p267. and pGSVLacZ groups (*p<0.05 vs pGSVLacZ).

Fig.6b Vascular resistance at rest and after administration of nitroprusside of the internal iliac artery in ischemic side (*p<0.05 vs pGSVLacZ).

Fig.7a Effect of pMJ35, p267, and pGSVLacZ on capillary density (/mm^2) at day 30 post-transfection (*p<0.001 vs pGSVLacZ group, #p<0.01 vs p267 group).

Fig.7b Effect of pMJ35, p267, and pGSVLacZ on capillary / muscle fiber ratio at day 30 post-transfection (*p<0.001 vs pGSVLacZ group and p267 group).

Fig.8 Evaluation of each muscle perfusion (ml/min/100g) of the ischemic limb in pMJ35, p267. and pGSVLacZ groups (*p<0.05 vs pGSVLacZ).

Fig.9 Dose-dependent effect of pMJ35 for angiogenesis.

a; Dose effect of pMJ35 on angiographic score (*p<0.001 vs 0 μg).

b; Dose effect of pMJ35 on calf blood pressure ratio (*p<0.001 vs 0 μg).

c; Dose effect of pMJ35 on capillary density (/mm^2) (*p<0.001 vs 0 μg).

d; Dose effect of pMJ35 on muscle perfusion assessed microsphere (ml/min/100g) (*p<0.05 vs 0 μg).

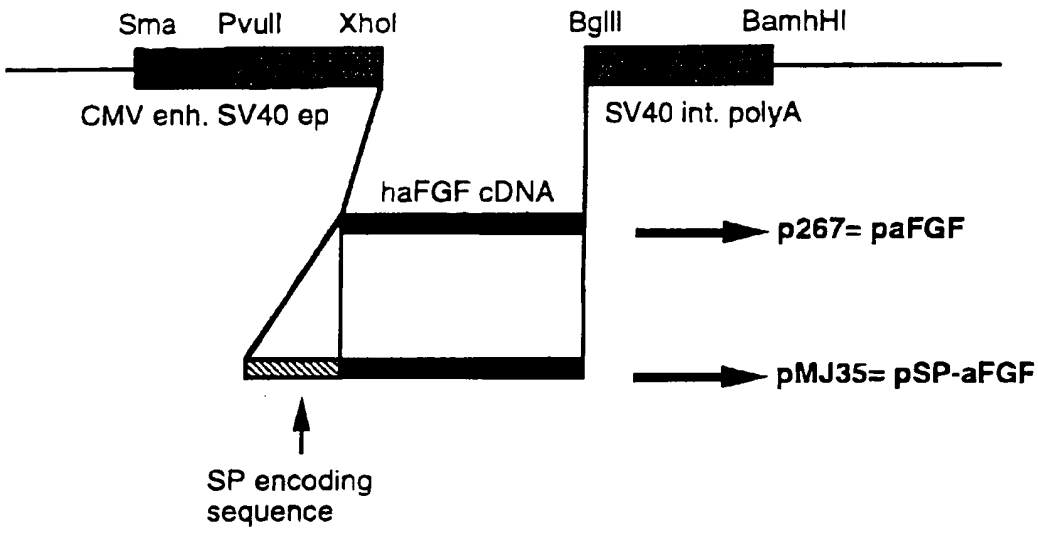

Sma
PvuII
XhoI
BglII
BamhHI
CMV enh. SV40 ep
SV40 int. polyA
haFGF cDNA
p267= paFGF
pMJ35= pSP-aFGF
SP encoding sequence

Fig. 1

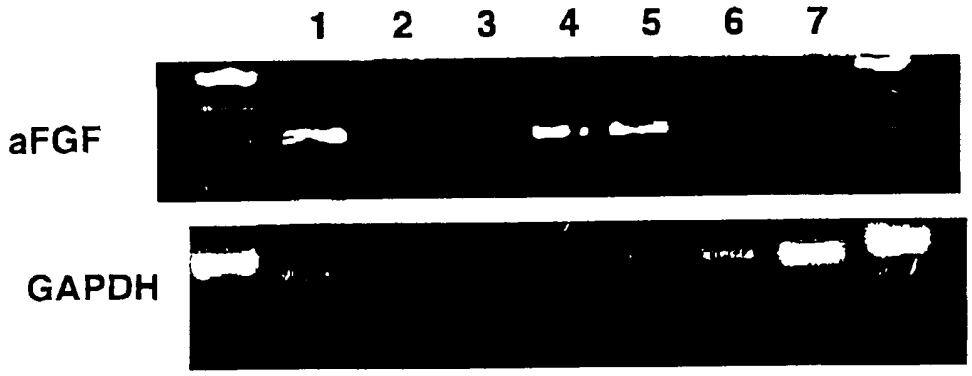


Fig. 2 a

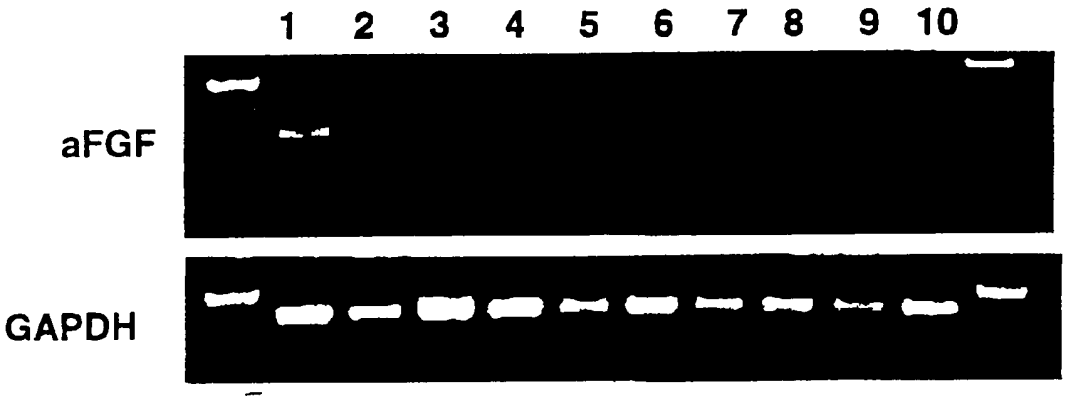


Fig. 2 b

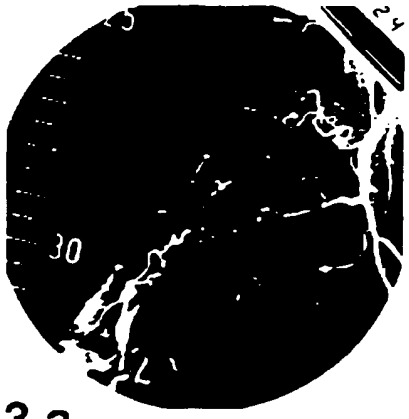


Fig-3 a

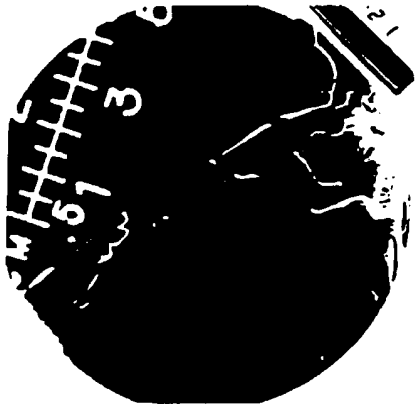


Fig-3 b

Fig-3 c

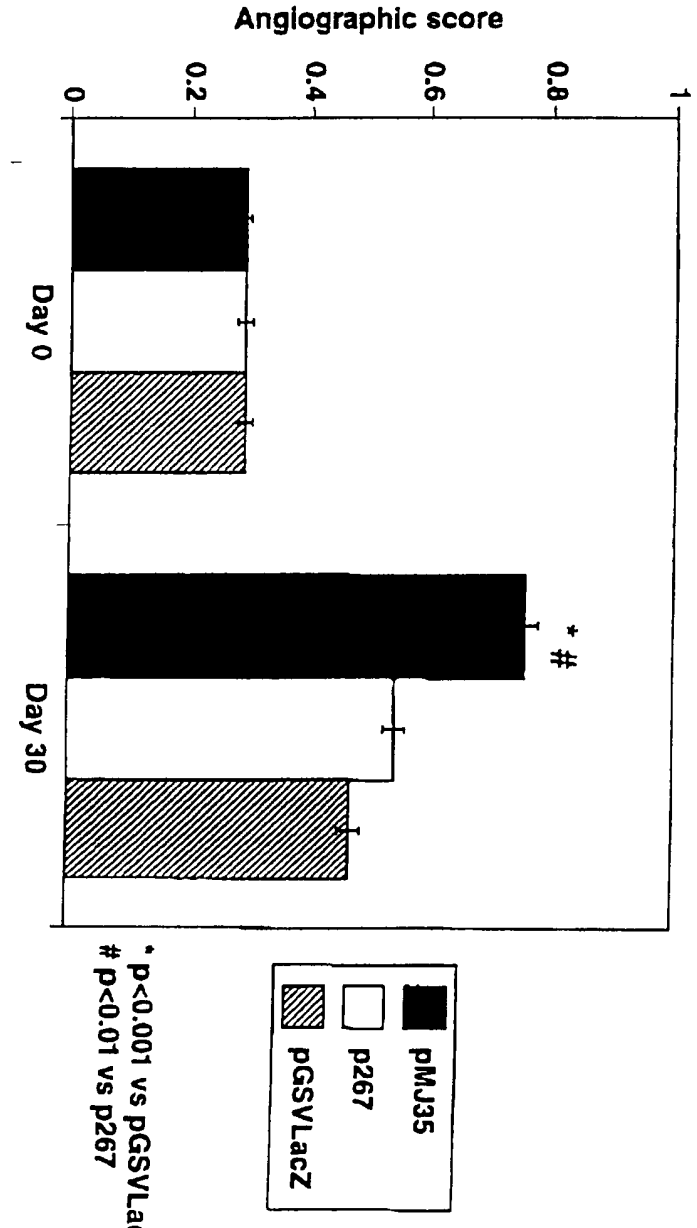

Angiographic score
0
0.2
0.4
0.6
0.8
1
Day 0
Day 30
* #
pMJ35
p267
pGSVLacZ
* p<0.001 vs pGSVLacZ
p<0.01 vs p267

Fig.4

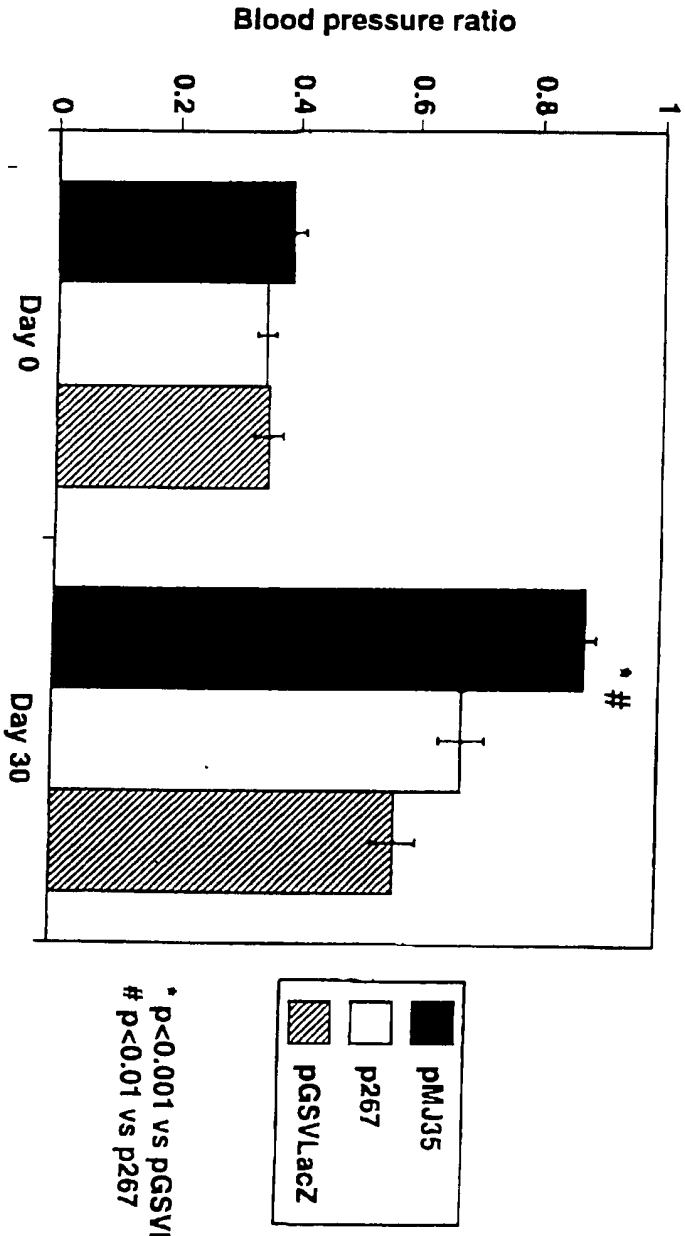

Blood pressure ratio
0
0.2
0.4
0.6
0.8
1
Day 0
Day 30
*
#
pMJ35
p267
pGSVLacZ
* p<0.001 vs pGSVLacZ
p<0.01 vs p267

Fig.5

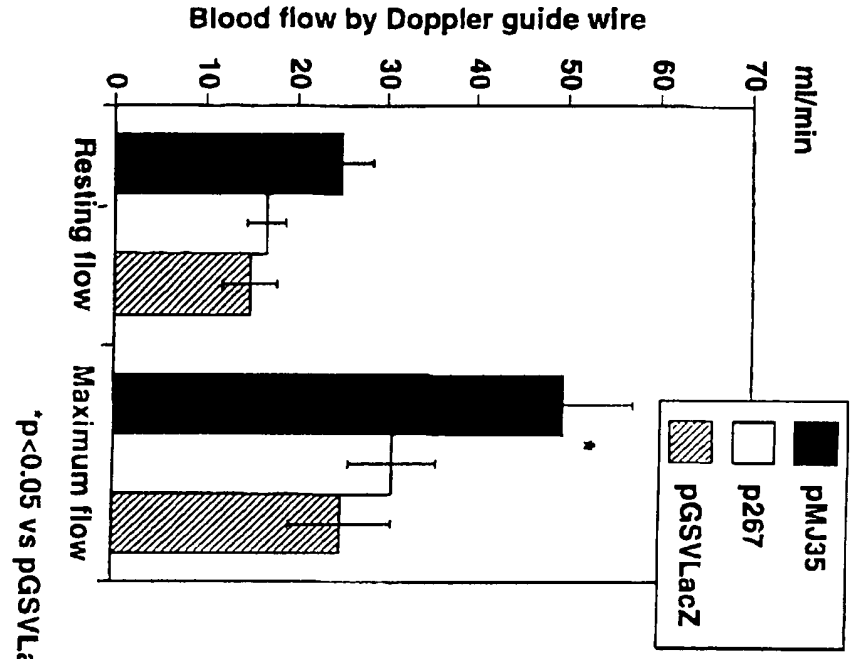

Blood flow by Doppler guide wire
0
10
20
30
40
50
60
70
ml/min
Resting flow
Maximum flow
*
*p<0.05 vs pGSVLacZ
pMJ35
p267
pGSVLacZ

Fig. 6a

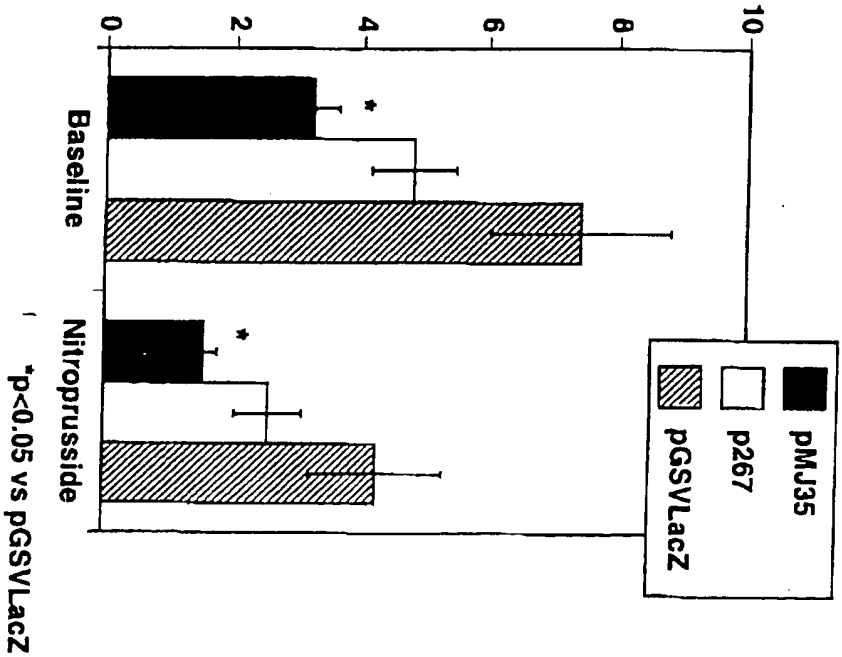
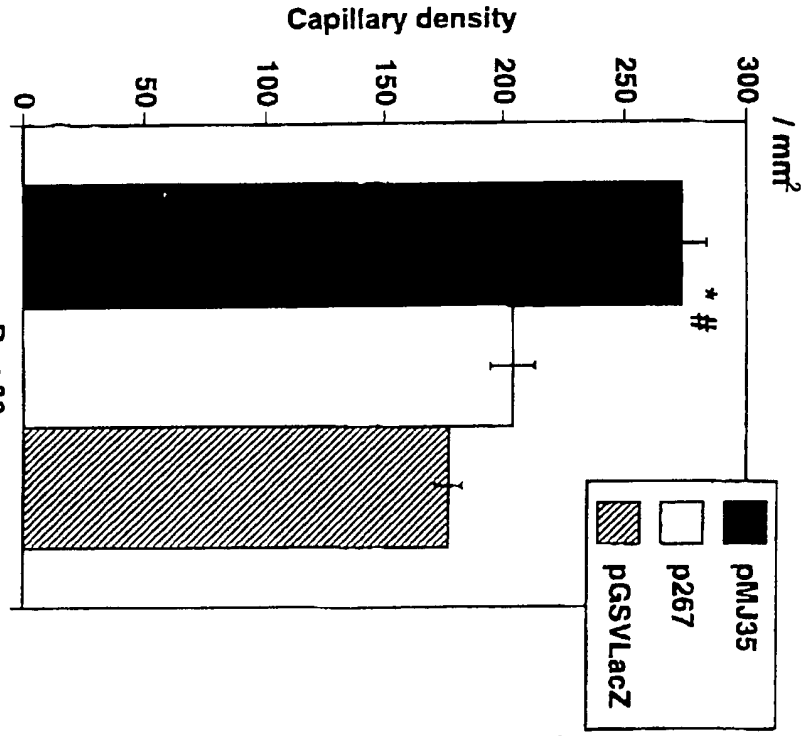
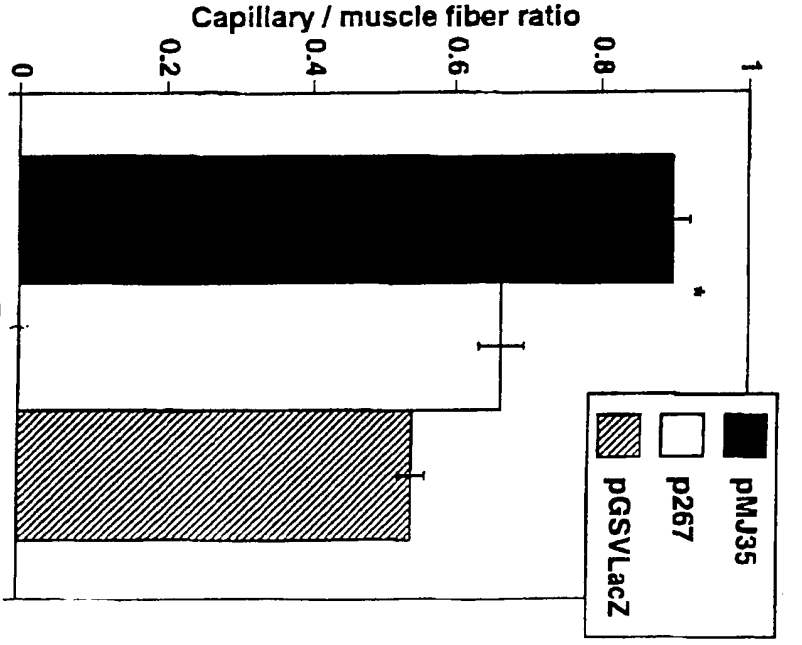

Vascular resistance
0
2
4
6
8
10
Baseline
Nitroprusside
*
*
*p<0.05 vs pGSVLacZ
pMJ35
p267
pGSVLacZ

Fig. 6b



* p<0.001 vs pGSVLacZ
p<0.01 vs p267

Fig. 7a



* p<0.001 vs pGSVLacZ
p267

Fig. 7b

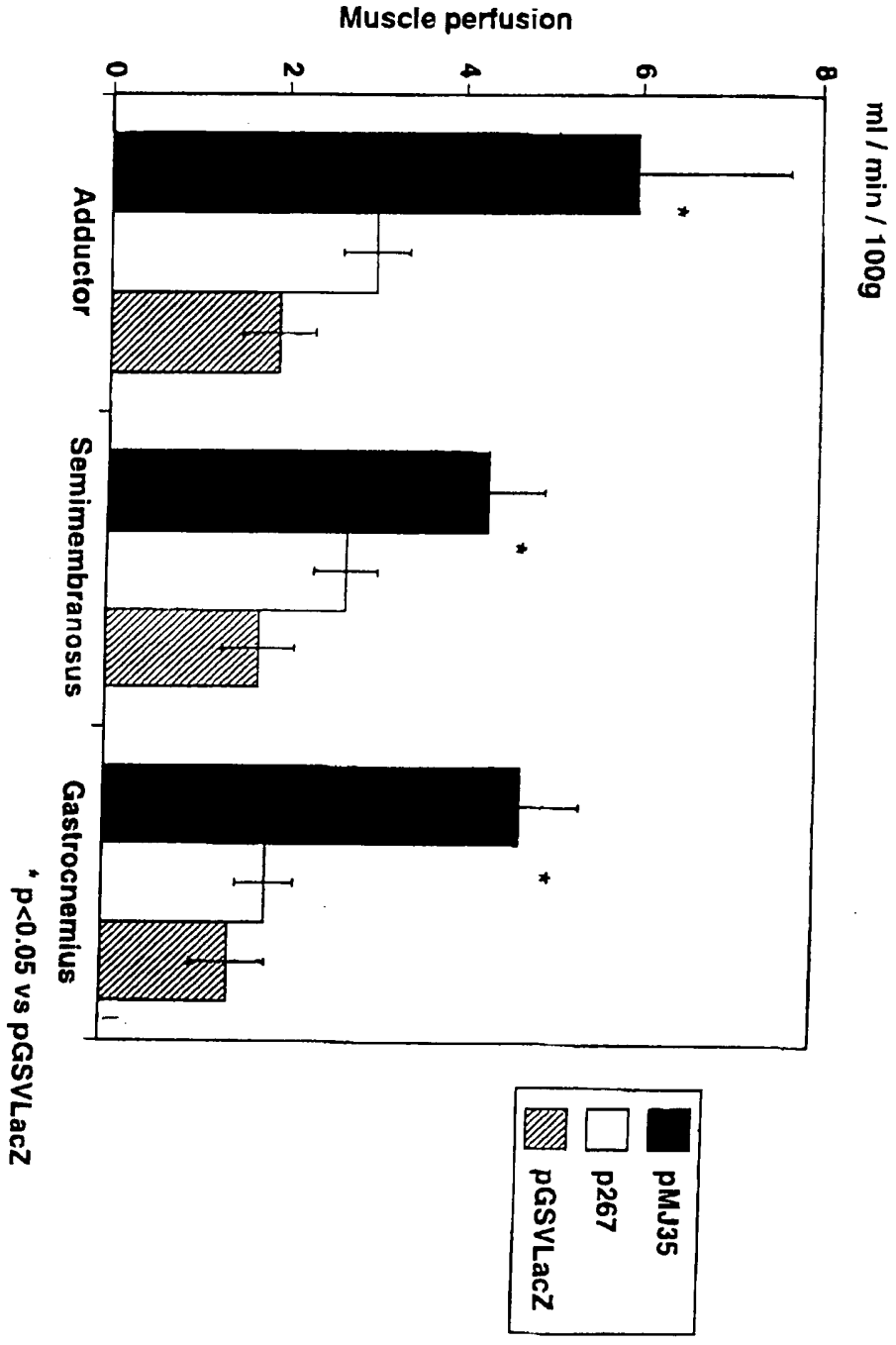

Muscle perfusion
0
2
4
6
8
ml / min / 100g
Adductor
Semimembranosus
Gastrocnemius
*
*
*
pMJ35
p267
pGSVLacZ
* p<0.05 vs pGSVLacZ

Fig. 8

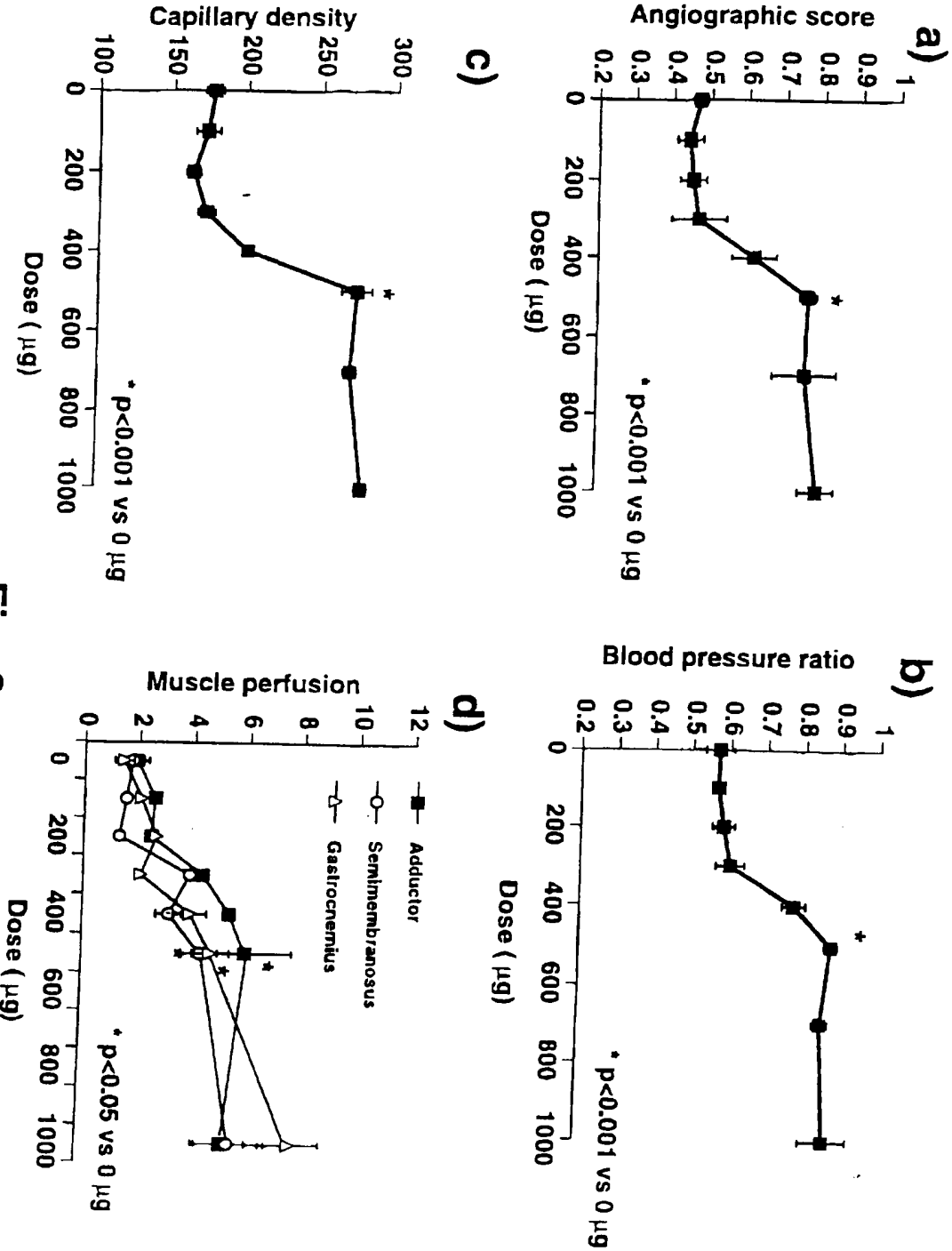

a)
Angiographic score
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
0
200
400
600
800
1000
Dose (µg)
* p<0.001 vs 0 µg
b)
Blood pressure ratio
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
0
200
400
600
800
1000
Dose (µg)
* p<0.001 vs 0 µg
c)
Capillary density
100
150
200
250
300
0
200
400
600
800
1000
Dose (µg)
* p<0.001 vs 0 µg
d)
Muscle perfusion
0
2
4
6
8
10
12
0
200
400
600
800
1000
Dose (µg)
* p<0.05 vs 0 µg
Adductor
Semimembranosus
Gastrocnemius

Fig. 9

Arterial Gene Transfer of Acidic Fibroblast Growth Factor for Therapeutic Angiogenesis in vivo: Critical Role of Secretion Signal in Use of Naked DNA

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Abstract

Background Previous studies have demonstrated that heparin-binding growth factors including acidic (aFGF) and basic (bFGF) fibroblast growth factor could promote angiogenesis. However, in vivo, the role of aFGF, that is lack of secreted signal originally, in vascular response has been difficult to determine. Therefore, we investigated the hypothesis that the gene transfer with naked aFGF DNA encoding secret signal sequence could achieve therapeutic angiogenesis in vivo.

Methods and results At ten days following surgical induction of unilateral hindlimb ischemia, New Zealand white rabbits were randomized to be performed intra-arterial gene transfer via a hydrogel balloon catheter in the internal iliac artery of the ischemic limb; 500 μg of pMJ35 (secreted type aFGF DNA)(n=10), 500 μg of p267 (non-secreted type aFGF DNA)(n=10), or 500 μg of pGSVLacZ (n=10). At day 30 post-gene transfer, pMJ35 transfectans had more angiographically visible collateral (angiographic score=0.76±0.02) vs p267 (0.55±0.02, $p<0.01$) vs LacZ (0.47±0.02, $p<0.001$). Calf blood pressure ratio for pMJ35 was 0.88±0.02 vs 0.68±0.04 for p267 ($p<0.01$) and 0.57 ±0.04 in LacZ ($p<0.001$). Capillary density (per square mm) was also superior in pMJ35 group (274±10) vs p267 (204±9, $p<0.01$) and LacZ (177±6, $p<0.001$). Flow to ischemic limb measured by intra-aortic Doppler wire at day 30 post-transfer showed that maximum flow (ml/min) after administration of nitroprusside was highest in pMJ35 group (49.6±7.6, $p<0.05$) vs LacZ (25.0±5.7). Perfusion of ischemic muscles, including adductor, semimembranosus, and gatrocunemius muscle, assessed by colored microsphere were also highest in pMJ35 group. RT-PCR disclosed human aFGF mRNA in transfected arteries of pMJ35 but not LacZ up to 21 days post-gene transfer.

Conclusion Optimal outcome for therapeutic angiogenesis following arterial gene transfer of naked aFGF DNA depends on the presence of secret signal sequence, as paracrine effects of secreted protein.

Introduction

Ischemic diseases represent the major cause of morbidity and mortality, and revascularization such as angioplasty or bypass surgery is undertaken commonly. However, revascularization of small or diffuse arteries is usually unsuccessful and restenosis or reocclusion after revascularization of relatively large arteries are still problem. Under these conditions, therapeutic strategies designed to argument native collateral vessel blood flow represent desirable means.

Acidic fibroblast growth factor (aFGF), which is mitogenic for endothelial cells, induces the components of vascular growth in vitro and stimulates the angiogenesis in vivo [1-3]. In addition, aFGF may play a role in the differentiation of the mesoderm during development [4] and shares sequence similarity with several oncogenes [5]. It is also reported that aFGF may both promote repair of damaged endothelium and inhibit the accompanying intimal thickening [6]. However, unlike most polypeptide growth factors, the structure of the aFGF translation product dose not possess an apparent classical secretory signal sequence [7-8] and aFGF open reading frame is flanked by termination codons [7]. A secreted form of the aFGF gene was derived by ligation of the signal sequence from the human fibroblast interferon to the first residue (methionine) of the encoded aFGF [9]. Though the site-specific transfection efficiency using catheter is relatively low, the gene encoding secreted protein may achieve therapeutic effect.

Accordingly, in the current study, we investigated the hypothesis that site-specific transfection of the plasmid encoding aFGF with secreted sequence could stimulate angiogenesis and modulate the hemodynamic deficit in chronic ischemic limb model, compared with the plasmid encoding aFGF without secreted sequence and control plasmid. The outcome of these in vivo experiments, performed in a rabbit model, resulted in therapeutic angiogenesis; evidence for this therapeutic endpoint included augmented

development of collateral vessels, marked reduction of hemodynamic deficit and increased capillary density.

Methods

Animal model

We used a rabbit ischemic hindlimb model that has been described previously [10], to investigate the angiogenic response. All protocols were approved by St. Elizabeth's Institutional Animal Care and Use Committee. Thirty male New Zealand White rabbis weighting 4-4.5 kg (Pine Acre Rabbitry, Norton, MA) were anesthetized with a mixture of ketamine (50 mg/kg) and acepromazine (0.8 mg/kg) following premedication with xylazine (2.5 mg/kg). A longitudinal incision was then performed, extending inferiorly from the inguinal ligament to a point just proximal to the patella. The limb in which the incision was performed - right versus left - was determined at random at the time of surgery by the operator. Through this incision, with surgical loops, the femoral artery was dissected free along its entire length; all branches of the femoral artery, including the inferior epigastric, deep femoral, lateral circumflex and superficial epigastric arteries, were also dissected free. After further dissecting the popliteal and saphenous arteries distally, the external iliac artery as well as all above arteries were ligated with 4.0 silk (Ethicon, Sommerville, NJ). Finally, the femoral artery was completely excised from its from its proximal origin as a branch of the external iliac artery, to the point distally where it bifurcates into the saphenous and popliteal arteries. The excision of the femoral artery results in retrograde propagation of thrombus to the origin of the external iliac artery. Consequently, blood flow to the ischemic limb is dependent upon collateral vessels which may originate from the internal iliac artery.